EXHIBIT

A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No.: 9295
LEE *et al.*	Art Unit: 1633
Appl. No.: 10/521,313	Examiner: Hill, Kevin Kai
§371 Filing Date: January 14, 2005; PCT Filed: July 15, 2003	Atty. Docket: 2298.0080002/EJH/BNC
For: **HER-2 NEU DNA VACCINE HAVING ANTI-CANCER ACTIVITY**	

Declaration of [Chang-Yuil Kang] Under 37 C.F.R. § 1.132

Mail Stop Amendment

Commissioner for Patents
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Sir:

The undersigned, **Chang-Yuil Kang**. residing at **Shingu Bless-valley Apt. 101-1006, Bangbae-dong, Seocho-gu, 137-851 Seoul, Republic of Korea**, declares and states as follows:

1. I am a co-inventor of above-captioned U.S. Patent Application No. 10/521.313 ("the '313 application") entitled "Her-2 Neu DNA Vaccine Having Anti-cancer Activity." which has a §371 filing date of January 14, 2005. which is a U.S. National Phase application of PCT/KR03/01400. filed July 15. 2003. which claims priority benefit to two Korean applications. KR 10-2002-0041764. filed July 16. 2002. and KR 10-2003-0038012, filed June 12, 2003.

2. I am currently employed by **Seoul National University**, where I hold the position of **professor**. My credentials are provided in the *curriculum vitae* that is attached to this declaration as **Exhibit A**. As seen from my attached *curriculum vitae*, I have published several papers related to **immunological researches and development of vaccines and immunotherapies** and I am involved in numerous professional and scientific societies related to **immunological researches**. Based on my education and experience, I have expertise in the field of vaccine research and development **and immunology**.

3. I have reviewed and am familiar with the Office Action dated April 22, 2008 ("the Office Action"), issued by the U.S. Patent and Trademark Office in the present application, and the claims, as being filed by amendment with this declaration.

4. In the Office Action, the Examiner asserts that the specification is not enabling for the disclosed method of preventing or treating mammalian subjects, including humans.

5. In making this declaration, it is my opinion that one of ordinary skill in the field of DNA vaccines as of July 16, 2002, the effective priority date of the present application, would have been enabled to practice the claims of the present application given the state of the art as of the filing date and the teachings of the specification.

6. It is my opinion that the specification, in combination with knowledge in the art at the time of filing, teach one of ordinary skill in the art how to make and use the

claimed invention without undue experimentation. In particular, the animal model used to test the DNA vaccine of the current invention was commonly used in the art in preclinical studies of vaccines, and was viewed as reasonably predictive of similar results in mammals, including humans. I am also aware of several reports that were published after the application was filed which show DNA vaccines are safe and effective in mammals, including humans, as taught in the specification. In addition, on-going phase I clinical trials for Her-2 DNA vaccines support that the specification was reasonably predictive of the methods and uses for Her-2/neu DNA vaccines in humans. Therefore, it is my opinion that the Examiner is in error, and I present herein the rationale upon which I base that conclusion.

7. The use of mice injected with cancer cells is a commonly used preclinical model for testing cancer vaccines, including protein and DNA vaccines, as shown by the following articles published prior to the effective priority date of the present application. In Conry *et al.*, "A Carcinoembryonic Antigen Polynucleotide Vaccine has in vivo Tumor Activity" *Gene Therapy 2*: 59-65 (1995) ("Conry 1995") **(Exhibit B)**, mice were inoculated with 2 x 10^5 syngeneic MC-38-CEA-2 cells by s.c. injection over the flank. *Id.* at 64. Tumors were measured by caliper in two dimensions, and the tumor volumes were calculated. *Id.* Conry *et al.* demonstratec that a CEA polynucleotide vaccine can immunoprotect against challenge with syngeneic CEA-transduced colon carcinoma cells. *Id.* at 62. Based on these preclinical results, a phase I clinical trial in patients with colorectal carcinoma was approved by the University of Alabama at Birmingham Institutional Review Board and the National Institutes of Health Recombinant DNA

Advisory Committee. *Id.* A report in 2002, published after the priority date of the current invention, described safety and efficacy results in humans using the DNA vaccine described in Conry 1995. *See* Conry *et al.*, "Safety and Immunogenicity of a DNA Vaccine Encoding Carcinoembryonic Antigen and Hepatitis B Surface Antigen in Colorectal Carcinoma Patients" *Clinical Cancer Research 2782*(8):2782-2787 (2002) ("Conry 2002") (**Exhibit C**). This and other first-generation clinical trials indicate that DNA vaccination is safe and well tolerated. *Id.* at 2786. Treatment with pCEA/HBsAg induced lymphoproliferative responses to CEA in 4 advanced stage disease patients. *Id.* at 2184-2185; Figure 1. Although the CEA-specific lymphoproliferative responses were not associated with objective tumor regression or sustained declines in circulating CEA, based on these results, additional Phase I/II trials with CEA polynucleotide immunization were proposed by the authors, particularly in patients with less advanced disease. *Id.* at 2186.

In Irvine *et al.*, "Cytokine Enhancement of DNA Immunization Leads to Effective Treatment of Established Pulmonary Metastases" *J Immunol. 156*(1): 238–245 (1996) ("Irvine") (**Exhibit D**), DNA-based immunization as a potential cancer treatment strategy was tested by injection of CT26 cells expressing a model tumor-associated Ag in BALB/c mice. BALB/c mice were injected i.v. with 2×10^5 CT26.CL25(β-gal$^+$) cultured tumor cells to induce pulmonary metastases. On day 17 after tumor injection, mice were killed and pulmonary metastases were enumerated. Irvine *et al.* showed that immunization with a pCMV/β-gal plasmid administered by particle-mediated gene delivery (gene gun) prevented the growth of pulmonary metastatic tumors and was

effective in active immunotherapy, particularly when administered with cytokine adjuvants following DNA administration.

In Penichet *et al.*, "A Recombinant IgG3-(IL-2) Fusion Protein for the Treatment of Human HER2/neu Expressing Tumors" *Hum. Antibodies 10*(1):43-9 (2001) ("Penichet") (**Exhibit E**), an anti-HER2/neu human IgG3 fusion protein containing interleukin-2 (IL-2) fused at its carboxyl terminus was tested in mice expressing Her-2-CT26 cells. To investigate *in vivo* anti-tumor activity, 10^6 CT26-HER2/neu cells were injected subcutaneously into the right flank of BALB/c mice. *Id.* at 44. Tumor growth was monitored and measured by caliper. *Id.* Penichet *et al.* showed that treating mice with anti-HER2/neu IgG3-(IL-2) caused significant retardation in the growth of CT26-HER2/neu tumors. *Id.* at 46. Even in the absence of complete tumor eradication in the mice, the authors concluded from these preclinical studies that "our results suggest that an anti-HER2/neu IgG3-(IL-2) fusion protein containing human IL-2 may be an effective therapeutic in patients with tumors overexpressing HER2/neu." *Id.* at 47.

Similarly, in the current invention, BALB/c mice were injected with Her2-CT26 cells. The specification describes that the Her-2/neu expressing syngeneic transfectoma Her-2-CT26 cells were prepared by transduction of CT26 cells with cDNA encoding human Her-2/neu. *See* Specification at [0073] - [0074] (all Specification page numbers are based on the published '313 application, US2006/0074038). BALB/c mice were injected with 5 x 10^4 Her2-CT26 cells either s.c. on the flank or i.v. *See* Specification at [0083]; [0098]. Tumors were measured by caliper in two dimensions, and the tumor volumes were calculated. *Id.* The results show that the DNA vaccines of the invention were able to induce both antibody and CD4+ T cell responses. Preventative and

therapeutic benefits of the DNA vaccine of the current invention were observed in the BALB/c-Her2-CT26 mouse model.

8. In my view, the examples of the specification describe results obtained in an animal model that reasonably correlates with an expectation of success in humans. In particular. the BALB/c-Her2-CT26 mouse model was used to test the preventative and therapeutic effects of the Her-2/neu DNA vacc.nes of the current invention. Preventive model tumor experiments were performed by challenging immunized mice with Her2-CT26 tumor cells. In mice immunized with the DNA plasmids of the invention, both Her-2/neu-specific antibody responses and Her-2/neu-specific CTL responses were induced. *See* Specification at [0095]-[0096]; [0115]-[0116]. Further, anti-tumor immunity against the Her2-CT26 tumor cells in pre-vaccinated mice was shown. All of the animals injected with vector alone developed palpable tumors; however, tumors were completely suppressed in mice injected with DNA vaccines of the current invention. *See* Specification at [0098]-[0099]; [0109]-[0110]. Therapeutic model experiments were performed by challenging the mice with tumor cells first, and then delivering i.m. injections of the DNA plasmids of the invention. When mice were exposed to metastatic tumor cells, treatment with the DNA vaccines of the current invention was shown to improve survival. *See* Specification at [0102]-[0105]; [0111]. The preventive and therapeutic anti-tumor activities of the truncated Her-2/neu DNA plasmids of the invention were promoted by co-injection of DNA encoding a cytokine as an adjuvant (either on a separate plasmid or bicistronic plasmid), resulting in decreased tumor growth and prolonged survival of vaccinated mice. *See* Specification at [0120]; [0124].

9. In my opinion, persons of ordinary skill in the art would consider the induction of CTL and antibody responses in the animal model used in the current invention a reasonable indicator of anti-tumor immunity in humans. The present application has provided working examples where i.m. administration of the claimed truncated Her-2/neu DNA vaccine according to a preset immunization schedule was effective at inducing CTL and antibody responses in mice both pre-injection and post-injection with human Her-2/neu expressing murine tumor cells (Her-2-CT26). Those of ordinary skill in the art were aware that DNA vaccines could elicit antibodies, CD4+ T cell responses and, importantly, CD8+ T cell responses in animals. *See*, e.g., Wang *et al.*, "Induction of Antigen-Specific Cytotoxic T Lymphocytes in Humans by a Malaria DNA Vaccine" *Science 282*:476-480 (1998) (**Exhibit F**). As of the priority date of the current invention, it was also known that induction of anti-tumor immunity, in part, involves CTL responses. Irvine at 238.

10. As of the time of filing, several studies had shown that antibody and CTL responses in mice could be recapitulated in other mammals, including humans. *See*, e.g., Wang, **Exhibit F**; Calarota *et al.*, "Cellular Cytotoxic Response Induced by DNA Vaccination in HIV-1-infected Patients" *The Lancet 351*:1320-1325 (1998) (**Exhibit G**); and Boyer et al., "Protection of Chimpanzees from High-dose Heterologous HIV-1 Challenge by DNA Vaccination" *Nature Med. 3*(5):526-532 (1997) (**Exhibit H**). In Wang, the work done in rodents and primates was used to develop a plan for manufacturing and testing the efficacy of a Malaria DNA vaccine in humans. Wang at

476. Wang discloses that as in the rodent model, the magnitude of CTL response that was seen in humans following DNA vaccination was considerably higher than the response generally seen in humans exposed to irradiated sporozoites or to natural infections. *Id.* at 479. In view of previous studies showing HIV-1 DNA vaccination to generate immune responses in animal models, Calarota *et al.* tested the efficacy of DNA vaccination in humans. Calarota at 1320. In Calarota, plasmid DNA that expressed HIV-1 regulatory genes *nef*, *rev*, and *tat* induced HIV-1-specific CTL reactivity in five of nine immunized individuals; three of the remaining individuals had transient responses and one did not react. *Id.* at 1323. These results show that results in mice tested with DNA vaccines were used by persons of ordinary skill in the art to develop DNA vaccines in humans. Furthermore, the results seen in humans were similar to those observed in mice. It is my opinion that, as of the priority date results obtained using the animal model of the instant specification reasonably correlated with an expectation of success in humans. Thus, induction of CTL and antibody responses in mice was reasonably predictive of similar effects in other mammals, including humans.

11. In addition to working examples, the specification includes guidance for administration, formulation and dosage of the plasmid constructs, which a person of ordinary skill would find sufficient to make and use the claimed invention. *See, e.g.*, Specification at [0064] - [0068]. Specifically, the specification describes a suitable dosage range from about 0.01 to 10 mg/kg/day, and further describes dosing to include a multiple dose schedule. *Id.*

12. The specification provides a clear road map to allow the ordinary artisan in the field of immunology to practice the invention without undue experimentation. Thus, given the explicit disclosure of specific *in vivo* working examples in the specification using a mouse model that reasonably correlates to mammals, including humans, it is my opinion that one skilled in the art would be able to make and use the claimed invention without undue experimentation.

13. In addition to the above teachings, it is my opinion that what the specification teaches a person of ordinary skill has been further confirmed in the literature. As shown below, DNA-based vaccines were well developed, or in progress, for a variety of disease conditions. For example, Malaria and HIV-1 DNA vaccines had been tested in rodent models and subsequently shown to elicit immune responses in humans. *See, e.g.*, Wang, **Exhibit F** and Calarota, **Exhibit G** (both discussed above).

14. The following exemplary references also establish the state of the art as of the effective priority date.

(a) Ulmer *et al.*, "DNA Vaccines," *Current Opinion in Immunology* *8*:531-536 (1996) ("Ulmer") (**Exhibit I**). Ulmer is a review article on DNA vaccine development. Ulmer reports that the continued expansion of the breadth of preclinical infectious and noninfectious disease targets successfully tested in animal models was important for bringing DNA vaccine technology closer to clinical application. These include viral targets, parasitic diseases, bacterial diseases and cancer (against which the first clinical trials for DNA vaccines were initiated during 1995). *Id.* at 532.

(b) Lodmell *et al.*, "DNA Immunization Protects Nonhuman Primates Against Rabies Virus," *Nature Medicine 4*:949-952 (1998) ("Lodmell") (**Exhibit J**). Lodmell discusses the advantages of DNA vaccines over conventional vaccines. *Id.* at 949. Lodmell cites ease of construction, ability to induce a full spectrum of long-lasting humoral and cellular immune responses, high temperature stability and low cost for their mass production. *Id.* Lodmell reports that immunization with a plasmid DNA vaccine encoding the glycoprotein of the rabies virus elicits protective immunity against lethal challenge in monkeys. *Id.* at 951.

(c) Agadjanyan *et al.*, "DNA Plasmid Based Vaccination Against the Oncogenic Human T Cell Leukemia Virus Type 1," *Current Topics in Microbiology and Immunology 226*:175-192 (1998) ("Agadjanyan") (**Exhibit K**). Agadjanyan is a review article that discusses vaccination strategies against human T cell leukemia virus type 1 ("HTLV-1"). Agadjanyan at 175. The article states that HTLV-1 has been demonstrated to be the causal agent of adult T cell leukemia and HTLV-1 associated myelopathy. *Id.* The article also reports that efforts have been successful in eliciting neutralizing antibodies as well as CTL activity in rodents and nonhuman primates with HIV-1 envelope expressing constructs. Agadjanyan at 180.

(d) Lee *et al.*, "Therapeutic Vaccine for Lymphoma" *Yonsei Medical Journal 48*(1): 1-10 (2007) ("Lee") (**Exhibit L**), is a review article that describes various therapeutic vaccines that have been tested for lymphoma prior to the effective priority date of the current invention. The unique antigenic determinants, idiotype (Id), of the tumor-specific immunoglobulin expressed on the surface of malignant B-cells can function as a tumor-specific antigen and had been exploited as a target for active

immunotherapy. *Id.* at 1. According to Lee, preliminary studies in mice and humans showed that a DNA vaccine for lymphoma was immunogenic when combined with adjuvant. Lee at 5-6, *citing* King *et al.*, "DNA Vaccines with Single-chain Fv Fused to Fragment C of Tetanus Toxin Induce Protective Immunity against Lymphoma and Myeloma" *Nat Med.* 4(11):1281-6 (1998) ("King") (**Exhibit M**). King describes a DNA vaccine containing a single-chain Fv, the V(H) and V(L) genes used to encode the individual Id determinants of each tumor assembled as single chain Fv (scFv), derived from B-cell tumors. Fusion of a pathogen-derived gene encoding fragment C (FrC) of tetanus toxin to the C-terminus of the scFv sequence led to a substantial promotion of antibody response and induced anti-idiotypic response in mice, and that the mice were protected against challenge with syngeneic B-cell tumor cells. *Id.* at 1281-1282. The authors also discuss that the ability of DNA vaccines containing fused scFv-FrC genes to induce protection against tumor challenge will encourage the planned clinical trial against lymphoma. *Id.* at 1285.

15. Additional work that was done after the effective filing date of the present application illustrates that the invention claimed in the present application was enabled as of the priority date of the present application. The references described below show that the teachings of the present application were reasonably predictive that the Her-2/neu DNA vaccine would be effective for preventing/treating spontaneous tumors. In addition, examples of DNA vaccines, including Her-2/neu DNA vaccines, are currently being tested in humans for cancer treatment, as was described in the specification of the instant application.

16. A recent example provides further support that rodent models for DNA vaccines, specifically Her-2/neu DNA vaccines, are likely to predict safe and effective results in humans. *See* Smorlesi *et al.*, "Evaluation of Different Plasmid DNA Delivery Systems for Immunization against HER2/neu in a Transgenic Murine Model of Mammary Carcinoma" *Vaccine 24*: 1766-1775 (2006) ("Smorlesi"), Smorlesi was previously cited by the Examiner in the Office Action dated April 22, 2008. Smorlesi describes a Her-2/neu DNA vaccine which was used to treat spontaneous tumor formation in a Her-2/neu transgenic model. Specifically, the DNA plasmid vaccine comprises a nucleotide sequence encoding an extracellular and transmembrane region of Her-2/neu under the control of the CMV early promoter. *Id.* at 1767. Smorlesi describes the effect of the DNA vaccine on the incidence and the growth of spontaneous tumors in FBVneu-T transgenic mice. *Id.* at 1769, Figure 1. These data indicate that the results shown in the present application, *e.g.*, decreased tumor formation in mice injected with Her-2/neu expressing cancer cells, were predictive that the Her-2/neu DNA vaccine would be effective for preventing/treating spontaneous tumors.

17. More recently, work that was initially tested in mice is now in clinical trials in humans. These studies further illustrate that mouse studies were reasonably predictive for therapeutic effect in humans. For example, phase 1 clinical trials show that HIV-1 and Ebola Virus DNA vaccines are safe and immunogenic in humans. *See* Graham *et al.*, "Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIV-1 DNA Candidate Vaccine" *J. Infect. Dis. 194*(12):1650-1660 (2006) ("Graham") (**Exhibit N**) and Martin

et al., "A DNA Vaccine for Ebola Virus Is Safe and Immunogenic in a Phase 1 Clinical Trial" *Clinical and Vaccine Immunology 13*(11):1267-1277 (2006) ("Martin") (**Exhibit O**), respectively.

18. Several Her-2 DNA vaccine trials have also been initiated. *See* Wei *et al.*, "The "A, B and C" of Her-2 DNA Vaccine Development," *Cancer Immunol. Immunother.* February 14, 2008 [Epublication ahead of print] ("Wei") (**Exhibit P**). Wei is a review article that describes the development of Her-2 DNA vaccines. According to Wei, the "C" phase of vaccine development are clinical trials, including a discussion of three Her-2 DNA clinical trials which are in progress. In particular, Wei describes a pilot clinical trial testing pVAX-E2A, conducted at the Karolinska Institute, Stockholm, Sweden, in stage IV breast cancer patients. The trial is titled "Vaccine immunization with nucleic acid coding for the gene Her-2/neu together with low doses GM-CSF (Leucomax*) and IL-2 (Proleukin*) as adjuvant in patients with metastatic breast carcinoma." *Id.* The study showed no adverse effects. *Id.* Thus, results shown in the specification of the current invention using the truncated Her-2/neu DNA construct and cytokine adjuvant of the invention to treat mice exposed to Her-2/neu tumor cells were reasonably predictive of the similar safety and efficacy in humans. In addition, in a current phase I study for a Her-2 DNA vaccine, the DNA vaccine is being administered intramuscularly as a series of 5 injections (2.5 mg/injection), every other week over a 94 week duration. *See* ClinicalTrials.gov Identifier: NCT00647114 at ClinicalTrials.gov (last visited, July 11, 2008) (**Exhibit Q**). The i.m. administration and dosage currently being tested in the NCT00647114 clinical trial are similar to the administration and dosage described in the

specification for the truncated Her-2/neu plasmid constructs of the current invention. Thus, the on-going Her-2 DNA vaccine clinical trials show that the specification of the current invention was reasonably predictive of therapeutic and preventative methods and uses of the claimed invention in humans.

19. I conclude that for at least the reasons stated above that the specification, in combination with teachings known in the art at the time the priority document was filed, teach a person of ordinary skill in the art to make and use the claimed invention without undue experimentation for methods of preventing or treating mammalian subjects, including humans.

20. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,

Date: Sept. 22, 2008 Chang Yuil Kang

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EXHIBIT

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Publications(International)

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Invited speaker at professional meetings

1. Functional analysis of various NKT ligands as adjuvants for the development of vaccine and immunotherapeutics, Postech-Catholic Bio Medical Engineering Institute, 08/07/09.
2. Technical development inducing NKT cell activation for treatment of cancer and virus disease, Korea Biotech R&D Group, 07/06/12-07/06/13.
3. Activation of NKT cells by alpha-galactosylceramide treatment licenses lamina propria dendritic cells to abrogate the oral tolerance, Rheumatism Research Center, 07/06/28-07/06/30.
4. Activation of NKT cells by alpha-galactosylceramide treatment licenses lamina propria dendritic cells to abrogate the oral tolerance, The American Association of Immunologists, 07/05/18-07/05/22.
5. 4-1BB engagement costimulates NKT cell activation and enhances airway hyperreactivity and airway inflammation mediated by NKT cells, The American Association of Immunologists, 07/05/18-07/05/22.
6. Strategy of HPV vaccine development, National Cancer Center, 07/03/16.
7. Cell based immunotherapy, Seoul National University Bio-Max Institute Korea Bio-Hub Center, 07/02/28.
8. 4-1BB engagement costimulates NKT cell activation and enhances airway hyperreactivity and airway inflammation mediated by NKT cells, The Korean Association of Immunobiologists, 07/04/13-07/04/14.

9. Regulatory T cells; immunobiology of regulatory T cell, The Korean Association of Immunobiologists, 07/04/14.
10. Combined chemoimmunotherapy can efficiently break self-tolerance and induce anti-tumor immunity in a tolerogenic murine tumor model, H. Lee Moffitt cancer center & research institute, 07/01/25-07/01/28.
11. aGalCer-loaded antigen expressing B cells induce a full spectrum of anti-tumor immunity, The Korean Association of Immunobiologists, 06/11/10.
12. A new cell-based immunotherapeutic approach: NKT cell-mediated B cell vaccine generates long-lasting cytotoxic anti-tumor immunity in vivo, The American Association of Immunologists, 06/05/12-06/05/16.
13. aGalCer-loaded antigen expressing B cells induce a full spectrum of anti-tumor immunity, The Korean Association of Immunobiologists, 06/11/10.
14. Activation of NKT cells by a-galactosylceramide treatment licenses lamina propria dendritic cells to abrogate the oral tolerance, The Korean Association of Immunobiologists, 06/11/10.
15. Combined chemoimmunotherapy can efficiently break self-tolerance and induce anti-tumor immunity in a tolerogenic murine tumor model, The Korean Association of Immunobiologists, 06/11/10.
16. Non-human primate studies of human Her-2/neu genetic vaccines, The Korean Association of Immunobiologists, 06/11/10.
17. Single intranasal immunization with inactivated influenza virus and a-galactosylceramide induces long-term protective immunity without redirecting antigen to the central nervous system, The Korean Association of Immunobiologists, 06/11/10.
18. Adenovirus-transduced B cells with NKT help generate effective antigen specific immune responses, The American Association of Immunologists, 06/05/12-06/05/16.
19. CD8a-CD11c+ DCs efficiently mediate the induction of immune responses when protein Ag and alpha-galactosylceramide are coadministered via intranasal route, The American Association of Immunologists, 06/05/12-06/05/16.
20. B cell based cancer immunotherapy, Yonsei University Medicine School Immune Disease Institute, 06/12/14.
21. Enhanced immunogenicity of DNA vaccine encoding mycobacterial Ag85B by codon usage optimization, The American Association of Immunologists, 05/03/31-05/04/05.
22. Alpha-galactosylceramide can act as a nasal vaccine adjuvant inducing protective immune responses against viral infection and tumor, The Korean Association of Immunobiologists, 05/04/15.
23. Alpha-galactosylceramide can act as a nasal vaccine adjuvant, The American Association of Immunologists, 05/03/31-05/04/05.
24. A new cellular vaccine using B cells co-pulsed with peptide and alpha-galactosylceramide induces long-lasting cytotoxic T lymphocyte immunity, International Cytokine Society, 05/10/27-05/10/31.
25. Alpha-galactosylceramide as a nasal vaccine adjuvant, International Cytokine Society, 05/10/27-05/10/31.
26. Linkage between the function and the phenotype of DC subsets from diverse lymphoid tissues, The Korean Association of Immunobiologists, 04/10/22-04/10/23.
27. NKT cell ligand alpha-galactosylceramide blocks the induction of oral tolerance by triggering dendritic cell maturation, The Korean Association of Immunobiologists, 04/04/09.
28. The CD8a-CD11b+DC mediates cross-tolerance toward intestinal antigen, The Korean Association of Immunobiologists, 04/10/22-04/10/23.
29. Complementary role of CD4+CD25+ regulatory T cells and TGF-beta in oral tolerance, The Korean Association of Immunobiologists, 04/04/09.
30. Split peripheral tolerance by CD40 signal: CD40 ligation blocks the cross-tolerance of CD8+ T cells whereas does not block CD4+ T cell tolerance in response to oral antigen, The American Association of Immunologists, 04/04/17-04/04/21.
31. Th1/Th2 and NKT cells in allergic inflammation, The Korean Academy of Athsma, Allergy and Clinical Immunology, 04/05/28-04/05/29.
32. Codon optimization effect of DNA vaccine encoding Mycobacterium Tuberculosis Ag85B on the immune Response, The Korean Association of Immunobiologists, 04/04/09.

33. Maturation of dendritic cells by NKT cell ligand blocks the induction of oral tolerance via providing costimulation, The American Association of Immunologists, 04/04/17-04/04/21.
34. Co-exposure to both allergen and NKT cell ligand is essential for the induction of asthma, The American Association of Immunologists, 04/04/17-04/04/21.
35. CD1d is not essential for the development of inflammatory response in the airway, The Korean Association of Immunobiologists, 04/10/22-04/10/23.
36. Induction and Maintenance of Immune Tolerance by Exogenous Antigen in the Absence of CD25+CD4+ Regulatory T cell in vivo, The Korean Association of Immunobiologists, 03/04/19.
37. Activation of APCs by CD40 ligation does not abrogate the induction of immune tolerance by oral antigen, American Association of Immunologists, 03/05/06-03/05/10.
38. The role of NKT cell in asthma, The Korean Association of Immunobiologists, 03/11/21-03/11/22.
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48. Antitumor immunity induced by plasmid DNA encoding secreted and membrane bound human Her-2/neu, The Korean Association of Immunobiologists,02/05/03/02/05/04.
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60. Identification of a domain containing B-cell epitopes in hepatitis C virus E2 glycoprotein, American Society for Biochemistry and Molecular Biology, 99/04/17-99/04/21.
61. Expression of human 4-1BB molecules on human T lymphocytes induced by allogenic mixed lymphocyte reaction, American Society for Biochemistry and Molecular Biology, 99/04/17-99/04/21.
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63. Kinetic analysis of oral tolerance: memory lymphocytes are refractory to oral tolerance, The Japaneses Society for Immunology, 99/12/01-99/12/03.
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70. The research of antibody development and specificity for immunoassay of Triazine, The Pharmaceutical Society of Korea, 96/10/25-96/10/26.
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77. Immunization with a soluble CD4-gp120 complex preferentially induces neutralizing anti-HIV antibodies directed to the conformational development epitopes of gp120, Korean Society for Molecular and Cellular Biology, 94/10/14.
78. Immunization with a soluble CD4-gp120 complex preferentially induces neutralizing anti-HIV antibodies directed to the conformational development epitopes of gp120, The Korean Society of Applied Pharmacology, 94/11/04.
79. Development of immunosuppressive anti-lymphocyte antibodies, The Korean Association of

Immunobiologists, 94/11/18.

80. Recent progress in the development of therapeutic antibodies, The Korean Association of Biological Response Modifiers, 94/06/24.
81. Soluble CD4-gp120 complex preferentially induces neutralizing anti-gp120 antibodies directed to the conformation dependent epitopes, The American Association of Immunologists, 94/04/24.

Patents

1) Application

1. Kang CY, Vaccine comprising monocytes or immature myeloid cells which were loaded with the ligand of natural killer T cell and antigen, 07/11/19, 2007-118066, Korea.
2. Kang CY, Vaccine comprising monocytes or immature myeloid cells which were loaded with the ligand of natural killer T cell and antigen, 07/11/28, PCT/KR2007/006057, PCT.
3. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 07/07/06, PCT/KR2006/001589, Japan.
4. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 07/04/30, 06 757 562.1, Europe.
5. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 07/05/01, 11/718391, USA.
6. Kang CY, Alpha-galactosylceramide derivatives, pharmaceutically acceptable salts thereof, preparation method and pharmaceutical composition for the immune adjuvant containing the same as an active gradient, 07/12/27, PCT/KR2007/006889, PCT.
7. Kang CY, A vaccine composition comprising alpha-galactosylceramide as an adjuvant for intranasal administration, 08/01/11, 11/995505, USA.
8. Kang CY, A vaccine composition comprising alpha-galactosylceramide as an adjuvant for intranasal administration, 08/01/02, PCT/KR2006/001226, China.
9. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 2006.
10. Kang CY(Pangenomics, Viromed), Her-2/neu DNA anti-tumor vaccine, 2006.
11. Kang CY, A vaccine composition comprising alpha-galactosylceramide as an adjuvant for intranasal administration, 2006.
12. Kang CY, Alpha-galactosylceramide derivatives, pharmaceutically acceptable salts thereof, preparation method and pharmaceutical composition for the immune adjuvant containing the same as an active gradient, 2006.
13. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 2006.
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15. Kang CY(Pangenomics), Her-2/neu DNA anti-tumor vaccine, 2003, PCT/KR03/01400, PCT.
16. Kang CY(Pangenomics), Her-2/neu DNA anti-tumor vaccine, 2002.
17. Kang CY(LG Chemistry), anti-CD40 ligand monoclonal antibody possessing immune suppression activity, 2001.

2) Registration

1. Kang CY, B cell-based vaccine loaded with the ligand of natural killer T cell and antigen, 08/02/27, 10-0809873, Korea.
2. Kang CY, A vaccine composition comprising alpha-galactosylceramide as an adjuvant for intranasal administration, 2007, Korea.
3. Kang CY, Preparation method of surface antigen protein of hepatitis B virus and thereof hepatitis B vaccine, 2000.
4. Kang CY, Preparation method of surface antigen protein of hepatitis B virus and thereof hepatitis B vaccine, 1997.
5. Kang CY, Monoclonal antibody specific for human 4-1BB and cell line producing same, 1996.
6. Kang CY, Monoclonal antibody specific for human 4-1BB possessing immune suppressive activity, 1995.

Major Research Interests

- Cancer Vaccine
- Therapeutic Antibodies
- Immune Tolerance
- Asthma Immunopathogenesis

EXHIBIT

AB

Gene Therapy (1995) 2, 59–65

A carcinoembryonic antigen polynucleotide vaccine has in vivo antitumor activity

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We have constructed a plasmid DNA encoding the full-length cDNA for human carcinoembryonic antigen (CEA) driven by the cytomegalovirus early promoter/enhancer and demonstrated that this plasmid can function as a polynucleotide vaccine. The immune response elicited by the CEA polynucleotide vaccine is dose and schedule dependent. There appears to be a threshold dose of 50 µg capable of inducing CEA-specific lymphoblastic transformation, lymphokine release, and antibody response. Doses of 10 µg were significantly less effective. When 50-µg doses are employed, thrice weekly or weekly vaccination schedules more reliably elicit CEA-specific immune responses by day 43 than does an every-3-weeks schedule. Furthermore the CEA polynucleotide vaccine can immunoprotect against challenge with syngeneic CEA-transduced colon carcinoma cells as early as 3 weeks after the first vaccination. Studies are ongoing to demonstrate the ability of CEA polynucleotide vaccination to treat pre-existing syngeneic mouse colon and breast carcinomas expressing human CEA.

Keywords: carcinoembryonic antigen; polynucleotide vaccine; genetic immunization; gene therapy; tumor; cancer

Introduction

There is growing interest in active specific immunotherapy for cancer [1, 2]. The identification and cloning of several tumor-associated antigens [3, 4, 5] has made possible a new generation of recombinant tumor vaccines utilizing such vectors as vaccinia virus [4, 6, 7]. More recently, direct intramuscular injection of plasmid DNA has been examined in the context of infectious diseases as a novel approach to vaccination, hereafter referred to as 'polynucleotide vaccination' [8, 9, 10]. This approach to gene transfer was discovered in 1990 when myofiber cells in the mouse were shown to express foreign genes that had been injected in the form of naked plasmid DNA [11]. A polynucleotide vaccine, encoding influenza A nucleoprotein, administered to mice produced influenza nucleoprotein-specific antibodies and cytolytic T cells as well as protection from subsequent challenge with influenza A virus [8]. This vaccine strategy has induced neutralizing antibodies to a human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein in mice [10] and non-human primates [9].

We have recently reported that administration of a polynucleotide vaccine encoding the cDNA for human carcinoembryonic antigen (CEA) to mice can induce an immune response specific for CEA [12]. Carcinoembryonic antigen is probably the most extensively characterized human tumor-associated antigen [13, 14]. This 180-kDa glycoprotein was originally thought to be present only in adenocarcinomas and fetal gut but subsequently was found in small amounts in normal colonic mucosa. The expression of CEA by adenocarcinoma cells is characteristic of human colon, breast and non-small cell lung cancer [13, 14]. The purpose of this report is to describe the effects of vaccine dose and schedule on immune response to CEA and to demonstrate that this form of gene therapy can produce *in vivo* antitumor effects, i.e. immunoprotection against tumor challenge with CEA transduced colon carcinoma cells.

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Results

Lymphoblastic transformation and lymphokine release data from five mice receiving 50-µg doses of the CEA polynucleotide vaccine (pCEA) thrice weekly for 12 injections is listed in Table 1. All five mice demonstrated dose-dependent lymphocyte proliferative responses to human CEA with peak stimulation ratios ranging from 2 to 30. All mice failed to respond to ovalbumin included as a control antigen with stimulation ratios ranging from 0.7 to 1.3, and mitogen responses were intact. All five mice also demonstrated dose-dependent lymphokine

Table 1 CEA-specific T cell response to polynucleotide vaccination with pCEA[a]

	Mouse 1	*Mouse 2*	*Mouse 3*	*Mouse 4*	*Mouse 5*
(a) Lymphoblastic transformation					
Control	340 ± 40[b]	520 ± 50	410 ± 50	230 ± 20	380 ± 40
CEA 100 µg/ml	3 300 ± 200	1 000 ± 200	4 600 ± 600	6 900 ± 300	11 000 ± 600
CEA 30 µg/ml	2 400 ± 100	840 ± 100	5 600 ± 200	6 500 ± 100	12 000 ± 600
CEA 10 µg/ml	1 100 ± 200	1 000 ± 100	3 800 ± 300	5 600 ± 200	9 000 ± 900
CEA 3 µg/ml	400 ± 40	440 ± 40	1 900 ± 200	3 400 ± 100	5 100 ± 300
OVA 100 µg/ml	420 ± 20	350 ± 60	520 ± 30	270 ± 50	450 ± 20
Con A	40 000 ± 2 000	18 000 ± 1000	28 000 ± 800	59 000 ± 1 000	35 000 ± 900
Pokeweed	34 000 ± 80	21 000 ± 1000	29 000 ± 1 000	33 000 ± 800	21 000 ± 900
(b) Lymphokine release (IL-2 and IL-4)[c]					
Control	430 ± 40[b]	890 ± 200	330 ± 40	450 ± 90	480 ± 100
CEA 100 µg/ml	4 600 ± 400	3 300 ± 500	4 700 ± 500	6 500 ± 600	6 600 ± 500
CEA 30 µg/ml	3 300 ± 200	1 900 ± 200	3 800 ± 700	3 700 ± 300	6 100 ± 300
CEA 10 µg/ml	4 300 ± 600	2 700 ± 300	2 700 ± 300	4 800 ± 400	5 600 ± 500
CEA 3 µg/ml	2 500 ± 600	1 200 ± 200	1 400 ± 100	3 700 ± 500	4 400 ± 200
OVA 100 µg/ml	440 ± 40	640 ± 200	550 ± 70	600 ± 80	530 ± 80

[a]Mice (five) received 50 µg of pCEA intramuscularly thrice weekly for 12 injections with spleens harvested on day 42.
[b]Values are mean c.p.m. of quadruplicate wells ± S.E.M.
[c]CTLL-2 assay which is specific for IL-2 and IL-4 in mice.

release (IL-2 and IL-4) to human CEA with peak stimulation ratios ranging from 4 to 15. Ovalbumin responses were again negative. Complete data from these five mice have been provided as mean counts per minute (c.p.m.) with the standard error to illustrate the reliability of these assays as well as the dose-dependent nature of the CEA-specific immune responses in the context of appropriate positive and negative controls. We have never seen positive cellular immune responses to CEA in unimmunized animals or animals immunized with control plasmids [12]. Although all experiments were conducted with the same panel of antigens and mitogens over the same range of concentrations, subsequent results have been provided as stimulation ratios for the maximum concentration of CEA (100 µg/ml) to facilitate reporting large quantities of data. In all instances, response to control ovalbumin was negative and mitogen responses were brisk.

To characterize the effect of CEA polynucleotide vaccine dose, groups of five mice were immunized weekly for six injections with varying doses of pCEA. All mice were sacrificed on day 43 and their splenic lymphoblastic transformation data is provided in Table 2. The 50-µg dose generated positive lymphoblastic transformation responses in five of five mice whereas all mice receiving 10-µg doses or 1-µg doses failed to respond.

Table 2 CEA-specific lymphoblastic transformation following varying doses of pCEA vaccine

pCEA Dose and schedule	*Stimulation ratio*[a] *Mouse 1*	*Mouse 2*	*Mouse 3*	*Mouse 4*	*Mouse 5*
1 µg weekly × 6	0.9	0.6	1.0	1.7	1.2
10 µg weekly × 6	1.3	1.0	0.7	1.1	1.2
50 µg weekly × 6	36.0	4.6	8.9	10.0	8.1

[a]Values are mean stimulation ratios from quadruplicate wells receiving 100 µg/ml of CEA.

To examine the effect of vaccination intensity, groups of five mice received 50-µg doses of pCEA thrice weekly, weekly, or every 3 weeks with immune response evaluated on day 43. The lymphoblastic transformation and lymphokine release responses are listed in Table 3. The thrice weekly and weekly schedules were equally efficacious with clearly positive CEA-specific lymphoblastic transformation occurring in four of five mice and five of five mice, respectively. Also, all five mice in both groups demonstrated lymphokine release to human CEA. The every-3-weeks schedule was less effective with only two mice clearly positive for lymphoblastic transformation, two mice with borderline responses, and one mouse negative. With regard to lymphokine release, three of five mice were positive, one was borderline, and one was negative.

Anti-CEA antibody response among mice vaccinated weekly for six injections with varying doses of pCEA is provided in Table 4a. Again, the 50-µg dose appears to be the threshold at which all five

Table 3 CEA-specific T cell response to varying schedules of pCEA vaccination

pCEA Dose and schedule	*Stimulation ratio* *Mouse 1*	*Mouse 2*	*Mouse 3*	*Mouse 4*	*Mouse 5*
(a) Lymphoblastic transformation[a]					
50 µg Q MWF × 12	9.7	2.0	11.0	30.0	29.0
50 µg weekly × 6	36.0	4.6	8.9	10.0	8.1
50 µg Q 3 weeks × 2	2.2	59.0	2.6	1.4	3.1
(b) Lymphokine release (IL-2 and IL-4)[b]					
50 µg Q MWF × 12	11.0	3.7	15.0	14.0	14.0
50 µg weekly × 6	14.0	7.6	4.8	8.0	5.1
50 µg Q 3 weeks × 2	1.7	8.5	3.5	6.4	2.3

[a]Values are mean stimulation ratios from quadruplicate wells receiving 100 µg/ml of CEA.
[b]Values are mean stimulation ratios from quadruplicate wells of CTLL-2 cells receiving supernatant from lymphocytes stimulated with 100 µg/ml of CEA.

Table 4 Humoral immune response to CEA[a]

pCEA Dose and schedule	*Mouse 1*	*Mouse 2*	*Mouse 3*	*Mouse 4*	*Mouse 5*
(a) pCEA Dose study					
1 μg weekly × 6	0	0	0	0	5
10 μg weekly × 6	59	11	88	0	36
50 μg weekly × 6	465	170	1013	295	545
100 μg weekly × 6	589	261	129	48	248
250 μg weekly × 6	137	43	99	112	361
(b) pCEA Schedule study					
50 μg Q MWF × 12	260	420	275	135	650
50 μg weekly × 6	465	170	1013	295	545
50 μg Q3 weeks × 2	0	0	0	0	200
200 μg Q3 weeks × 3	85	1070	345	0	335

[a]Results expressed as ng of [^{125}I]-CEA bound/ml of plasma (see Materials and methods). A positive assay is defined as being >3 S.D. above normal mouse serum and is >15 ng/ml.

mice become positive. Lower doses of 10 μg and 1 μg produced anti-CEA antibody responses in only three of five mice and none of five mice, respectively. Meanwhile, higher doses of 100 μg and 250 μg produced no apparent increment in anti-CEA antibody response compared with the 50-μg dose.

Table 4b lists the CEA-specific antibody response among groups of five mice receiving 50-μg doses of pCEA thrice weekly, weekly, or every 3 weeks with sera obtained on day 43. An additional group is included which received 200-μg doses of pCEA every 3 weeks for three injections with sera obtained on day 50. The thrice weekly and weekly schedules are equally efficacious with five of five mice in both groups demonstrating anti-CEA antibody on day 43. Among the mice receiving 50 μg of pCEA every 3 weeks for two injections (days 0 and 21), only one of five animals demonstrated anti-CEA antibody by day 43. However, the every-3-weeks schedule did achieve four of five mice positive for anti-CEA antibody by day 50 using a dose of 200 μg and three injections (days 0, 21 and 42). The anti-CEA antibody response correlates well with lymphoblastic transformation and lymphokine release data provided for the same mice in Table 3. Based on humoral and cellular immune response on day 43, the thrice weekly and

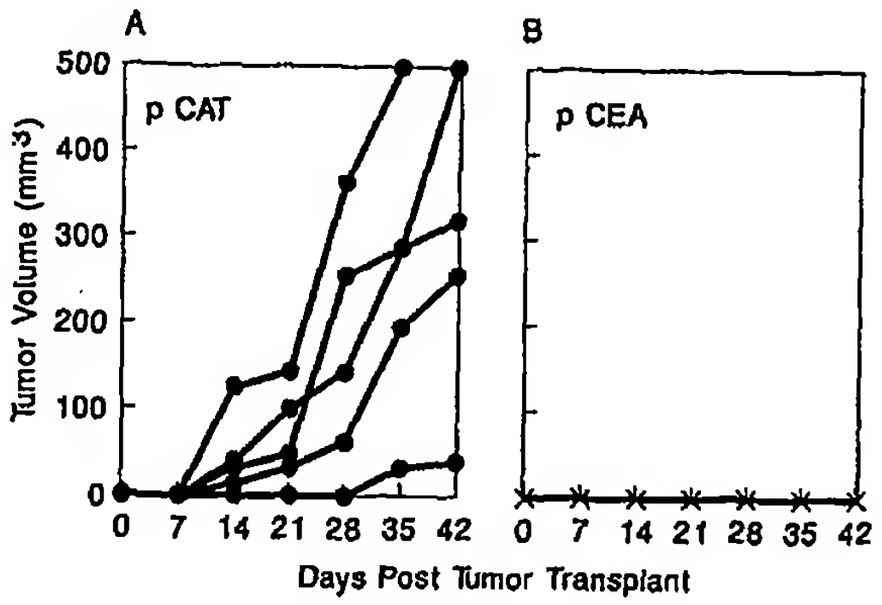

Figure 1 Effect of CEA polynucleotide vaccination on growth of a transplanted mouse adenocarcinoma cell line expressing human CEA. Five mice per group were vaccinated with 200 μg of either pCAT (A) or pCEA (B) every 3 weeks for three injections. Three weeks after the last vaccination MC-38-CEA-2 cells were transplanted by subcutaneous inoculation.

weekly schedules appear equivalent to one another and superior to the every-3-weeks schedule.

The ability of the CEA polynucleotide vaccine to immunoprotect against transplantation of syngeneic CEA-transduced colon carcinoma cells was examined. The initial study utilized our previously reported dose and schedule [12]. Groups of five mice were vaccinated with 200-μg doses of pCEA or a control plasmid encoding chloramphenicol acetyltransferase (pCAT) every 3 weeks for three injections. Seven days after the last vaccination, all ten mice were inoculated with 2×10^5 MC-38-CEA-2 cells. The results are illustrated in Figure 1. All five mice receiving the pCAT control plasmid developed readily measurable tumors within 42 days of tumor inoculation whereas none of five mice receiving pCEA developed tumors within 42 days. These mice receiving pCEA have been followed for 100 days with only one of five developing a tumor. Therefore, the CEA polynucleotide vaccine delivered every 3 weeks for three injections can generate a CEA-specific immune response by day 50 which is protective against tumor challenge.

We next examined the antitumor effects of the more intensive vaccine schedules at an earlier time point. Groups of ten mice were vaccinated with 50-μg

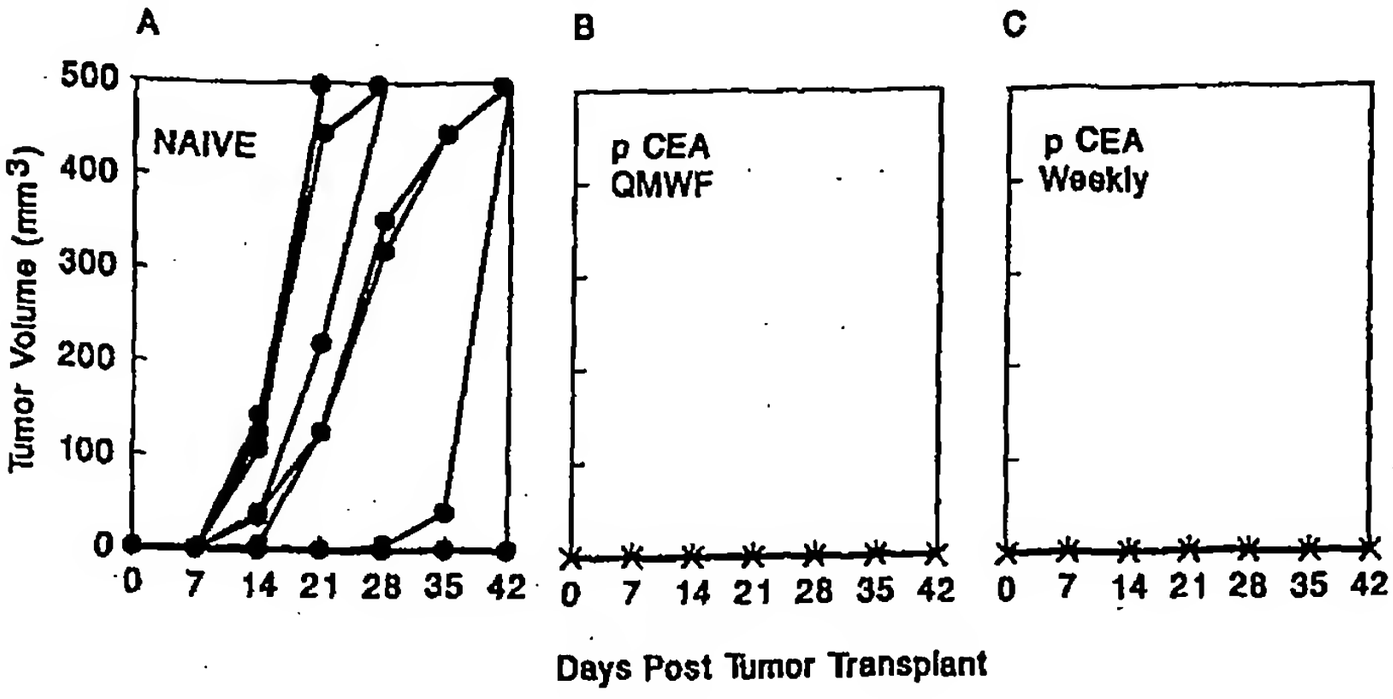

Figure 2 Effect of CEA polynucleotide vaccination on growth of a transplanted mouse adenocarcinoma cell line expressing human CEA. Ten mice per group were vaccinated with 50-μg doses of pCEA thrice weekly (B) or weekly (C) for 3 weeks. Naive mice (A) served as controls. On day 22, MC-38-CEA-2 cells were transplanted by subcutaneous inoculation. Seven of ten naive mice developed tumors with tumor growth curves for two mice overlapping.

doses of pCEA thrice weekly for eight injections or weekly for three injections followed by inoculation with MC-38-CEA-2 cells on day 22. Ten age matched naive mice received MC-38-CEA-2 cells on the same day as controls. The results are illustrated in Figure 2. Seven of ten naive mice developed readily measurable tumors by day 42 post-tumor transplant. However, none of the 20 mice receiving pCEA on either schedule developed tumors within 42 days. These mice have been followed for 10 weeks with evidence of tumor growth in only one of 20 mice receiving pCEA.

Discussion

We have constructed a plasmid DNA encoding the full-length cDNA for human CEA driven by the cytomegalovirus early promoter/enhancer (pCEA) and demonstrated that this plasmid can function as a polynucleotide vaccine when delivered intramuscularly [12]. Furthermore, the immune response elicited by the CEA polynucleotide vaccine is dose and schedule dependent. There appears to be a threshold dose of 50 µg capable of inducing CEA-specific lymphoblastic transformation, lymphokine release, and antibody response. Doses of 10 µg were significantly less effective with anti-CEA antibody responses induced in only three of five mice and no evidence of lymphoblastic transformation to CEA. These data correlate with observations of Manthorpe *et al.* [15] who examined luciferase gene expression in mice following intramuscular injection of plasmid DNA. Increasing the dose of plasmid DNA from 10 µg to 50 µg resulted in a 35-fold increment in luciferase expression whereas expression plateaued at doses above 50 µg. When 50-µg doses are employed, thrice weekly or weekly vaccination schedules more reliably elicit CEA-specific immune responses by day 43 than does an every-3-weeks schedule. This objective relationship between dose/schedule and immune response will facilitate the design and interpretation of polynucleotide vaccine clinical trials.

We have further demonstrated that the CEA polynucleotide vaccine can immunoprotect against challenge with syngeneic CEA-transduced colon carcinoma cells as early as 3 weeks after the first vaccination. This *in vivo* immunoprotection is similar to that described for a vaccinia-CEA tumor vaccine [6] and for infectious disease trials of polynucleotide vaccines against influenza and HIV [8–10]. To our knowledge, this represents the first report of *in vivo* antitumor effects by a polynucleotide vaccine in a solid tumor model. Based upon these preclinical studies, a phase I clinical trial in patients with colorectal carcinoma has been approved by the University of Alabama at Birmingham Institutional Review Board and the National Institutes of Health Recombinant DNA Advisory Committee.

Multiple animal model studies [2, 16] have demonstrated that immune response to tumor-associated antigens can have dramatic anti-tumor effects, but such treatment strategies rapidly lose efficacy as progressive tumor growth occurs. Thus, the induction of an immune response to tumor-associated antigens in humans is likely to have limited success in patients with obvious metastases; and its optimal application would be at the time of occult, micrometastasis as an adjunct to primary therapy.

Studies are ongoing to demonstrate the ability of CEA polynucleotide vaccination to treat pre-existing syngeneic mouse colon and breast carcinomas expressing human CEA. In addition to therapy of local tumor growth with the MC-38-CEA-2 model as previously described [6], we will employ a therapy model for micrometastases consisting of the 4T1 murine mammary carcinoma line previously utilized for vaccine studies [17]. The success of such 'therapy' models may be problematic in this treatment system since these are rapidly growing tumor models and the gene expression of polynucleotide vaccines reaches a maximum at 10–14 days after injection [15]. This time limitation would not be important in adjuvant trials of human breast or colon cancer since micrometastases generally produce clinically detectable metastases 1–2 years after primary tumor removal.

Three hypotheses could explain the mechanism whereby polynucleotide vaccination elicits a CEA-specific immune response. First, the myocytes could function as antigen presenting cells [18, 19] with intracellular synthesis of CEA followed by major histocompatibility complex (MHC) class I peptide display promoting T cell activation and cell surface expression of the transmembrane glycoprotein providing B cell activation. A second hypothesis calls for myocytes to simply provide an endogenous source of CEA to draining lymph nodes. There, intact CEA could be recognized by B cells as well as be processed by 'professional' antigen presenting cells (dendritic cells, macrophages and B cells) for presentation to T cells with appropriate costimulation. Alternatively, priming of the immune response may require a small quantity of CEA to reach the draining lymph nodes for antigen processing and presentation with appropriate costimulation. Thereafter, prolonged presentation of CEA by myocytes may serve to boost the immune response of memory T cells which are considerably less dependent on accessory cell costimulation than naive cells [20]. Studies are ongoing to test each of these hypotheses.

Polynucleotide vaccination offers many advantages over previous tumor vaccine strategies. It elicits both antibody and cell-mediated immune response using a nonreplicating vaccine without adjuvants, and intracellular synthesis of the tumor-associated antigen favors MHC class I peptide display considered pivotal to the generation of cytolytic T cells [21]. Such MHC class I display would be important whether the myocyte functions to prime the immune response or simply to boost the response of memory T cells. Gene expression in skeletal muscle following plasmid injection has been detected for up to 19 months after injection, with the foreign plasmid DNA appearing to remain episomal [22]. Persistent

expression of the encoded antigen would be expected to favor long-lived humoral and cellular immunity [23, 24] whether the myocyte functions as an antigen presenting cell or an endogenous source of CEA to draining lymph nodes. Finally, large quantities of purified DNA for vaccination can be prepared and standardized with relative ease compared with traditional protein purification techniques.

Materials and methods

Plasmid DNAs for vaccination

The gene for full-length human CEA [25] was used to construct an expression plasmid from the vector pcDNA3 (Invitrogen, San Diego, CA, USA), in which transcription is driven by the cytomegalovirus early promoter/enhancer [12]. As control, we utilized the plasmid DNA pcDNA-CAT (Invitrogen), which contains the chloramphenicol acetyltransferase reporter gene in the pcDNA3 vector. For simplicity, the resulting plasmids were designated pCEA and pCAT. Large-scale preparation of the plasmid DNAs was carried out by the procedure of alkaline lysis followed by cesium chloride density gradient centrifugation [26]. After extraction with 2-propanol to remove residual ethidium bromide, the DNA was precipitated in large lots (5 µg) and stored at –70°C as pellets. For experimental use, the DNA was reconstituted in sterile saline at a concentration of 5 mg/ml and stored in aliquots at –20°C for direct employment in injection/vaccination protocols. These specific methods were employed as published works had noted: (1) DNA of a high level of purity is most efficacious for eliciting an immunologic response by the direct injection method; and (2) individual batch-to-batch variations of DNA require the development of sufficient amounts of homogenous reagent for each distinct experiment (15). Quality control included the ability of pCEA to direct synthesis and cell surface expression of CEA, demonstrated through transduction of NIH 3T3 fibroblasts by the adenovirus–polylysine conjugate method [27] and immunohistochemical staining with the CEA-specific COL-1 monoclonal antibody [28].

Cells and reagents

Production and maintenance of the human CEA transduced MC-38-CEA-2 subline from the murine colonic adenocarcinoma cell line, MC-38 [29], has been previously described by us [25]. Before inoculation into animals, CEA expression was verified using murine monoclonal antibody COL-1 [29]. Cell lines CTLL-2 and NIH 3T3 were obtained from the American Type Culture Collection. Purified human CEA was obtained from hepatic metastases of human colonic adenocarcinoma (Vitro Diagnostics, Littleton, CO, USA) [6]. Ovalbumin, concanavalin A (Con A) and pokeweed mitogen were obtained from Sigma Chemical Company (St Louis, MO, USA). Polystyrene beads (6.4-mm) were obtained from Precision Plastic Ball (Chicago, IL, USA). G418 was obtained from GIBCO BRL (Grand Island, NY, USA).

Vaccination method

Six to eight-week-old C57BL/6 mice (Charles River Laboratories, Raleigh, NC, USA) were anesthetized with ketamine and xylazine by intraperitoneal injection. Tongues were pulled out of the mouth gently with forceps to allow a 28-gauge needle to penetrate the bulk of the tongue muscle. All polynucleotide vaccinations employed a constant 50-µl volume of normal saline with plasmid DNA concentration varying with dose. Preliminary studies by our laboratory [12] and others [30] have demonstrated that 50 µl is the most reliable volume for delivery into the tongue, and this volume does not impair the ability of animals to eat or drink following recovery from anesthesia. The tongue was selected to allow direct visualization of the striated muscle without necessitating a surgical procedure. Direct visualization of the muscle for injection ensures that the full dose of plasmid DNA is delivered into the muscle. Representative mice were routinely killed for necropsy and cultures to rule out intercurrent infections within the colony.

Experimental design

In order to characterize the effects of vaccine dose on immune response, groups of mice (n=5) received weekly injections of 1, 10, 50, 100 or 250 µg for a total of six doses and were then killed on day 43 for analysis of humoral and cellular immunity to CEA. To examine the effect of vaccination intensity (schedule and dose), groups of mice received 50-µg doses every 3 weeks for two injections, weekly for six injections, or thrice weekly for eight injections. Mice were killed on day 43 with humoral and cellular immune response to CEA determined.

We examined *in vivo* antitumor effects by tumor challenge in two studies. In the first, we used our previously described [12] dose and schedule of 200 µg of pCEA or pCAT control plasmid given on three occasions at 3-week intervals (600 µg total dose) with tumor challenge on day 50. To examine the *in vivo* activity of the more intense vaccine schedules, we vaccinated with 50-µg doses weekly for three injections (150 µg total dose) or thrice weekly for eight injections (total dose 400 µg) with tumor challenge on day 22.

Lymphoblastic transformation

This assay was performed as previously described [12, 6]. Stimulated wells received purified human CEA over a range of concentrations (3–100 µg/ml); ovalbumin (100 µg/ml) as a negative control antigen; or positive control mitogens, Con A (5 µg/ml) and pokeweed (2 µg/ml). The range of CEA concentrations described above provided optimal stimulation in our previous studies of pCEA immunized mice [12, 6]. The stimulation ratio was calculated as mean c.p.m. of the stimulated wells divided by mean c.p.m. of the control wells. A positive assay was defined as a stimulation ratio ≥2.0.

Lymphokine release

T cell responses were examined through biologic assessment of IL-2/IL-4 release as previously described [12]. Briefly, mononuclear cells were cultured exactly as above with the same panel of antigens or mitogens over the same range of concentrations with the exception that after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at –70°C. Supernatant IL-2 and IL-4 activity was quantified based on its ability to support proliferation of CTLL-2 cells sensitive to both cytokines [31]. Results were expressed as mean c.p.m. of quadruplicate wells. The stimulation ratio was calculated as mean c.p.m. of wells receiving supernatants from stimulated wells divided by mean c.p.m. of wells receiving supernatants from control wells. A positive assay was defined as a stimulation ratio ≥2.0. Standard curves were also generated for each assay using recombinant mouse IL-2 or IL-4.

Antibody assay

Antihuman CEA antibody was quantified using a double antigen immunoradiometric assay as previously described [12, 32]. Briefly, polystyrene beads were coated with purified human CEA (2 μg/bead) in phosphate buffered saline (PBS), washed three times with PBS containing 1% bovine serum albumin and stored in wash buffer at 4°C until use. Twenty microliters of mouse serum (normal control or post-vaccination) was diluted to 100 μl with PBS and incubated with a single coated bead (in duplicate) for 2 h on a laboratory oscillator at room temperature, washed with PBS and incubated with 100 μl of [^{125}I]-labeled human CEA (2 μg/ml) for 1 h (approximately 200 000 c.p.m./bead), rewashed with PBS and counted on an automatic gamma counter (ICN Micromedic Systems, Huntsville, AL, USA). Background non-specific binding of approximately 1% of the available [^{125}I]-CEA was subtracted from c.p.m. bound and the nanograms of CEA bound to the bead per milliliter of serum was calculated from the known specific activity of the [^{125}I]-CEA. A positive assay (>15 ng/ml) has been defined as exceeding three standard deviations above the mean value of ten normal mouse sera.

Tumor challenge

Mice were inoculated with 2×10^5 syngeneic MC-38-CEA-2 cells by subcutaneous injection in sterile PBS through a 20-gauge needle over the flank as previously described [6]. Tumors were measured by caliper in two dimensions, and the volumes were calculated using the formula (width^2 × length)/2 [6]. As few as 2×10^4 MC-38-CEA-2 cells produce tumors in 70–100% of naive mice (unpublished observations).

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Gene Therapy

EXHIBIT

AC

Advances in Brief

Safety and Immunogenicity of a DNA Vaccine Encoding Carcinoembryonic Antigen and Hepatitis B Surface Antigen in Colorectal Carcinoma Patients[1]

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Abstract

Despite an abundance of preclinical data, relatively little is known regarding the efficacy of DNA vaccination in humans. Here, we present results from a dose-escalation clinical trial of a dual expression plasmid encoding carcinoembryonic antigen (CEA) and hepatitis B surface antigen (HBsAg) in 17 patients with metastatic colorectal carcinoma. CEA was selected as a prototypic tumor-associated self-antigen, and the HBsAg cDNA was included as a positive control for immune response to the DNA vaccine without relying upon breaking tolerance to a self-antigen. Groups of 3 patients received escalating single i.m. doses of the DNA vaccine at 0.1, 0.3, and 1.0 mg. Subsequent groups of 3 patients received three repetitive 0.3- or 1.0-mg doses at 3-week intervals. A final group of 2 patients received three repetitive 2.0 mg doses at 3-week intervals. Toxicity was limited to transient grade 1 injection site tenderness, fatigue, and creatine kinase elevations, each affecting a minority of patients in a non-dose-related manner. Repetitive dosing of the DNA vaccine induced HBsAg antibodies in 6 of 8 patients, with protective antibody levels achieved in four of these patients. CEA-specific antibody responses were not observed, but 4 of 17 patients developed lymphoproliferative responses to CEA after vaccination. No objective clinical responses to the DNA vaccine were observed among this population of patients with widely metastatic colorectal carcinoma. Nevertheless, this pilot trial has provided encouraging human immune response data in support of this vaccine technology.

Introduction

Between 1990 and 1993, the administration of "naked" plasmid DNA encoding a specific protein antigen was shown to induce expression of the protein in mouse myocytes, elicit antibodies against the protein, and protect against influenza challenge via cytolytic T-cell responses against the expressed protein (1–3). Hundreds of publications since have reported the efficacy of DNA vaccines in small and large animal models of infectious diseases and cancer (4, 5).

DNA vaccination provides the following advantages over protein vaccines: (*a*) greater chemical stability; (*b*) relative ease of purification and characterization; (*c*) inherent adjuvant effects of unmethylated CpG dinucleotide motifs; (*d*) direct entry of antigen into intracellular MHC class I processing pathways facilitating CTL induction; and (*e*) intracellular antigen synthesis with posttranslational modification producing native tertiary antigen structure. Similarly, DNA vaccination provides advantages compared with recombinant viral vaccines as follows: (*a*) relative ease of construction, production and quality control; (*b*) less risk of insertional mutagenesis; (*c*) absence of vector-specific immune responses that limit the efficacy of booster immunizations; and (*d*) absence of risk related to recombinational events leading to pathogenic viruses.

Despite an abundance of preclinical data, relatively little is known regarding the efficacy of DNA vaccination in humans. Early clinical trials of DNA vaccines against infectious pathogens have provided mixed results. Trials conducted in HIV-infected individuals have been difficult to interpret because of preexisting immunity (6–8). A subsequent Phase I trial of a DNA vaccine encoding HIV antigens in 39 healthy volunteers induced antigen-specific lymphoproliferative responses but no antibody responses and only rare CTL responses (9). In the largest DNA vaccine trial reported to date, 219 healthy volunteers were randomized to receive a plasmid encoding influenza hemagglutinin or placebo (10). Virus-neutralizing and hemagglutination-inhibiting antibodies developed in only a minority of subjects. More encouraging results have emerged from the initial clinical trial of a DNA vaccine encoding a malarial antigen, the *Plasmodium falciparum* circumsporozoite protein (11). Persons receiving vaccines unequivocally developed antigen-specific, CD8-positive CTLs restricted by multiple HLA alleles, providing a foundation for further human trials of this potentially revolutionary vaccine technology.

In this report, we present results from a dose-escalation clinical trial of a dual-expression plasmid encoding CEA[3] and HBsAg in 17 patients with metastatic colorectal carcinoma.

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; HBsAg, hepatitis B surface antigen; PHA, phytohemagglutinin; S.R., stimulation ratio; rV-CEA, recombinant vaccinia virus encoding CEA.

CEA was selected as a prototypic tumor-associated self-antigen, and HBsAg cDNA was included as a positive control for immune response to the DNA vaccine uncomplicated by immunological tolerance to a self-antigen. CEA is a M_r 180,000 membrane-anchored glycoprotein expressed on the great majority of colorectal, gastric, and pancreatic carcinomas as well as ~50% of breast cancers and 70% of non-small cell lung cancers (12). CEA is also expressed, to a limited extent, in normal colonic mucosa and fetal digestive organs.

Our group has demonstrated that i.m. administration of plasmid DNA encoding human CEA induces CEA-specific humoral and cellular immune responses in mice and nonhuman primates as well as protection of mice against challenge with syngeneic colon carcinoma cells expressing human CEA (13–15). However, it is important to emphasize that human CEA is a foreign antigen in both mice and nonhuman primates, whereas humans should be immunologically tolerant to CEA because of its expression in fetal and normal adult tissues.

The goals of the present study were 3-fold: (*a*) to examine the safety of single and repetitive administration of the DNA vaccine over a range of 0.1–2.0 mg/dose; (*b*) to examine the efficacy of DNA vaccination in humans based upon immune response to the HBsAg control antigen; (*c*) to evaluate any CEA-specific immune responses or antitumor effects induced by the vaccine.

Materials and Methods

DNA Vaccine. We obtained the cDNA encoding full-length human CEA from J. Kanter, National Cancer Institute (16) and cDNA for the small and middle (S2.S) proteins of HBsAg from Robert Whalen (Centre National de la Recherche Scientifique, Paris, France; Refs. 17, 18). These two cDNAs were inserted into a eukaryotic expression vector that uses separate cytomegalovirus intermediate-early promoter/enhancers to regulate transcription of CEA and HBsAg. The plasmid was derived from the commercially available eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA). The plasmid contains two cytomegalovirus intermediate-early promoter/enhancers and bovine growth hormone polyadenylation signals flanking two multiple cloning sites. The pcDNA3 plasmid was modified by deletion of the neomycin resistance gene, the ampicillin resistance gene, and nonessential viral sequences (19). The Tn903 kanamycin resistance gene from pUC4K (Pharmacia, Piscataway, NJ) was inserted to allow selective propagation in *Escherichia coli*. The resulting plasmid was designated pCEA/HBsAg. *In vitro* expression and *in vivo* immunogenicity of pCEA/HBsAg in nonhuman primates has been reported previously (15). Clinical supplies of this construct (IND 6892) were produced under good manufacturing practices by Centocor, Inc. (Malvern, PA). The DNA vaccine was formulated in a citrate-buffered saline solution containing 0.25% bupivacaine-HCl, an amide-type anesthetic, as described previously (20). Three DNA vaccine concentrations of 0.05, 0.15, and 0.5 mg/ml were stored as 1-ml vials at −20°C and thawed at room temperature for 30 min immediately before injection.

Treatment Regimen. Seventeen patients with metastatic colorectal carcinoma, shown to express CEA by immunoperoxidase staining or by an elevated serum CEA level, were selected (21). Patients had an Eastern Cooperative Oncology Group performance status of 0–2 and no serological evidence of antibody to HBsAg or active hepatitis B infection. Therapy was initiated at least 4 weeks after prior chemotherapy or radiotherapy. All patients gave informed consent.

Each DNA vaccine dose consisted of bilateral i.m. injections using a volume of 1–2 ml into each deltoid muscle. Groups of 3 patients received escalating single doses of pCEA/HBsAg at 0.1, 0.3, and 1.0 mg total dose. Subsequently, groups of three patients received three repetitive 0.3 or 1.0-mg doses at 3-week intervals. A final group of two patients received three repetitive 2.0 mg doses at 3-week intervals. All patients underwent 9 weeks of clinical and immunological monitoring after their first DNA vaccine dose. Patients were examined 1 and 3 weeks after each immunization to assess the degree of inflammation at the inoculation site, regional adenopathy, or other signs of toxicity. A complete blood count, serum chemistries, and C-reactive protein level were obtained before each immunization as well as 7 and 28 days after each immunization. Serum CEA levels were obtained at 3-week intervals, and anti-double-stranded DNA antibody titers were checked before immunization and during the off-study evaluation on day 64. All patients were evaluated for evidence of objective antitumor response 9 weeks after the primary immunization.

Lymphoproliferative Assay. Fresh peripheral blood mononuclear cells obtained by Ficoll density gradient centrifugation were resuspended in complete medium consisting of RPMI 1640 supplemented with 10% pooled normal human AB serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics. Cells were added at 1.5×10^5 per well to 96-well, flat-bottomed plates. Stimulated cells were incubated in quadruplicate wells with baculovirus-derived recombinant human CEA (MicroGeneSys, Meriden, CT) over a range of concentrations (1–30 μg/ml); yeast-derived recombinant HBsAg (kindly provided by Merck, Sharp, and Dohme, West Point, PA) over a range of concentrations (1–30 μg/ml); BSA (30 μg/ml) as a negative control antigen; tetanus toxoid (Wyeth-Ayerst Laboratories, Paoli, PA) as a positive control antigen; PHA at 5 μg/ml as a positive control mitogen; or baculovirus-derived recombinant HIV gp160 (MicroGeneSys) as a control for proliferation induced by trace contaminants within the baculovirus recombinant CEA preparation. Control cells were cultured in complete medium alone. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 6 days, followed by an overnight pulse with 1 μCi/well of tritiated thymidine. Cells were harvested, and incorporated radioactivity was quantitated using a solid-phase beta scintillation counter (Matrix 9600; Packard Instrument Co., Downers Grove, IL). This solid-phase counter is ~3-fold less efficient than a liquid-phase scintillation counter such that raw cpm are 3-fold fewer than those seen with liquid counting. The S.R. was calculated as the mean cpm of the stimulated cells divided by the mean cpm of the control cells. A positive response was defined as a postimmunization S.R. >3 and at least 2-fold greater than the pre-immunization S.R. for a given antigen.

Antibody Response to HBsAg. Anti-HBsAg antibody responses were evaluated by the commercially available AUSAB ELISA (Abbott Laboratories, Chicago, IL), which detects antibodies to the small S protein of HBsAg. Anti-HBsAg antibody levels

Table 1 Patient characteristics

Patient no.	Age/Sex	Dosage (mg)	Performance status[a]	Metastatic disease	Serum CEA Pre-study (ng/ml)	Serum CEA week 9 (ng/ml)	Response[b]
1	71/M	0.1 × 1	1	Liver	960	3,923	P
2	62/F	0.1 × 1	0	Liver, Lymph Node	127	180	S
3	61/M	0.1 × 1	1	Lung, Lymph Node	893	1,819	P
4	62/M	0.3 × 1	1	Liver, Peritoneum	293	554	P
5	55/M	0.3 × 1	2	Liver, Peritoneum	3	7	P
6	59/F	0.3 × 1	1	Liver, Peritoneum	7	15	P
7	56/F	1.0 × 1	1	Lung, Liver	214	371	P
8	70/F	1.0 × 1	1	Lymph Node, Skin	166	332	S
9	54/M	1.0 × 1	1	Liver	311	446	P
10	49/F	0.3 × 3	1	Peritoneum	33	41	S
11	67/M	0.3 × 3	1	Liver	501	666	P
12	53/M	0.3 × 3	1	Liver, Lymph Node	822	969	P
13	73/F	1.0 × 3	1	Liver, Peritoneum	159	480	P
14	69/F	1.0 × 3	1	Lung, Liver	2,807	3,978	S
15	51/F	1.0 × 3	0	Lung	62	106	S
16	51/M	2.0 × 3	0	Lung, Liver	53	93	P
17	50/F	2.0 × 3	0	Lung, Liver	10	87	P

[a] Performance status on Eastern Cooperative Oncology Group Scale (0–4).
[b] Response based upon the day 64 off-study evaluation. S, stable disease; P, progressive disease.

≥10 mIU/ml in the Abbott ELISA correlate with protective immunity in humans (22). CEA-specific antibody responses were evaluated by ELISA and Western blot using methods described previously (23).

Results

Patient Characteristics. The clinical aspects of the trial are summarized in Table 1. The patients included 8 men and 9 women with a median age of 59 years (range, 49–73). All patients had metastatic colorectal carcinoma, and 16 of 17 had elevated serum CEA levels (>3 ng/ml) at the time of study entry. Five of 17 patients had stable disease at 9 weeks of follow-up, whereas the remaining 12 patients demonstrated disease progression. None of the patients demonstrated a sustained decrease in serum CEA levels after vaccination.

Toxicology. i.m. injection of the DNA vaccine produced transient grade 1 tenderness at the injection site in a minority of patients unrelated to dose. No signs of local inflammation, regional lymphadenopathy, or allergic reactions occurred. A few patients reported mild, transient fatigue, which was not dose related.

Transient grade 1 elevations of creatine kinase occurred in two patients 1–2 weeks after immunization in a non-dose-related manner. None of the patients developed anti-double-stranded DNA antibodies. C-Reactive protein levels, a marker of the hepatic acute phase protein response, were elevated in 8 of 17 patients before immunization, consistent with the diagnosis of advanced carcinoma (24). Elevated postimmunization values, which were greater than twice the pre-immunization value, occurred in 7 patients. C-Reactive protein levels typically peaked at or near the time of off-study evaluation, when patients were experiencing progressive metastatic carcinoma. Thus, the observed increase in acute phase reactants is consistent with progressive carcinoma and does not necessarily implicate inflammation or tissue damage as a consequence of DNA vaccination.

Grade 1–2 anemia developed in a non-dose-related fashion in 6 patients and was consistent with anemia of chronic disease in this patient population with metastatic colorectal carcinoma. Granulocytes have been shown to express non-specific cross-reacting antigen, raising the possibility that immunization against CEA could lead to autoimmune neutropenia (12, 25). Despite this theoretical concern, no consistent change in WBC counts or neutrophil counts occurred after vaccination. No deterioration in renal or hepatic function attributable to immunization was observed.

Lymphoproliferative Responses. Lymphoproliferative responses to CEA occurred in 4 of 17 patients after DNA immunization. The quotient of the postimmunization S.R. divided by the pre-immunization S.R. for baculovirus recombinant CEA and baculovirus recombinant HIV gp160 control protein is illustrated in Fig. 1 for these 4 patients. Postimmunization responses to CEA ranged from 2.6- to 44-fold greater than pre-immunization values. These lymphoproliferative responses appear CEA specific, because responses to HIV gp160 control protein did not increase after immunization. Details of the postimmunization lymphoproliferative responses in these 4 patients are provided in Table 2. CEA produced stimulation ratios ranging from 3.5 to 78. BSA and baculovirus recombinant HIV gp160 control proteins produced negative results with S.R.s ranging from 0.5 to 1.1 and from 0.5 to 2.7, respectively. PHA mitogen produced stimulation ratios >200 in all patients at all studied time points. CEA-specific lymphoproliferative responses occurred 6–9 weeks after primary immunization and showed no clear relationship to the dose or schedule of plasmid DNA immunization.

No detectable lymphoproliferative responses to HBsAg were observed in any of the 17 patients studied. Lymphoproliferative responses to tetanus toxoid and PHA mitogen were analyzed to determine the effect of progressive metastatic colorectal carcinoma upon lymphocyte function *in vitro*. Proliferative responses to PHA did not change significantly over 9 weeks

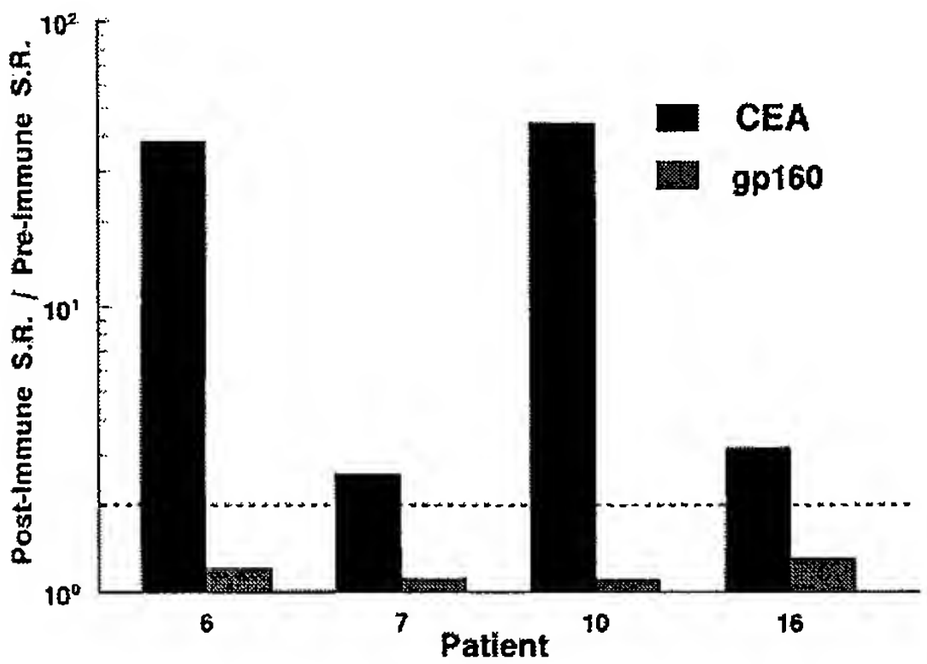

Fig. 1 Lymphoproliferative responses to CEA of patients immunized with pCEA/HBsAg. Values represent the quotient of the postimmunization stimulation ratio (S.R.) divided by the pre-immunization S.R. for a given antigen. The *dashed line* at 2 represents the threshold for a positive response. ■, baculovirus recombinant CEA protein; ▩, baculovirus recombinant HIV gp160 as a control protein.

of follow-up, despite disease progression in 12 of 17 patients. The mean ± SE S.R. in response to PHA pre-study and 9 weeks after primary immunization were 1060 ± 220 and 880 ± 130, respectively. Conversely, proliferative responses to a recall antigen, tetanus toxoid, diminished significantly during 9 weeks of follow-up. Six of 17 patients demonstrated lymphoproliferative responses to tetanus toxoid pre-study, but none of these 6 patients responded to tetanus toxoid at the time of off-study evaluation (day 64).

Antibody Responses. Anti-HBsAg antibody responses were evaluated by the commercially available AUSAB ELISA (Abbott Laboratories), which detects antibodies to the small S protein of HBsAg. All patients were negative for anti-HBsAg antibody pre-immunization as a criterion for study entry. Three of 9 patients receiving a single dose of pCEA/HBsAg developed HBsAg antibodies, but only 1 achieved a protective level exceeding 10 mIU/ml (patient 5, 157 mIU/ml at week 9). Repetitive dosing of pCEA/HBsAg induced HBsAg antibodies in 6 of 8 patients, and 4 of these patients achieved protective antibody levels. Among patients receiving repetitive doses of the DNA vaccine, HBsAg antibody responses showed a trend toward dose dependence, occurring in only 1 of 3 patients in the 300-μg dose group compared with 5 of 5 patients receiving 1–2-mg doses. None of the patients developed detectable CEA-specific antibodies.

Discussion

A major aim of the study was to examine the safety of single and repetitive administration of the DNA vaccine at total doses ranging from 0.1 to 6.0 mg. Toxicity was limited to transient grade 1 injection site tenderness, fatigue, and creatine kinase elevations, each affecting a minority of patients in a non-dose-related manner. This is consistent with other Phase 1 trials of DNA vaccines reporting occasional injection site erythema or tenderness and no systemic toxicity (5, 6, 8, 11, 26, 27). Thus, the upper limit of dosing for i.m. DNA vaccines appears to be determined predominantly by practical limits on the production of clinical grade plasmid DNA rather than toxicity.

A second aim of the study was to examine the efficacy of DNA vaccination in humans based upon immune response to the HBsAg control antigen. Repetitive dosing of pCEA/HBsAg induced HBsAg antibodies in 6 of 8 patients, with protective antibody levels achieved in 4 of these patients. HbsAg DNA immunization has had mixed success to date (28, 29). Particle-mediated delivery of another DNA vaccine encoding HBsAg to the epidermis failed to induce primary immune responses to HBsAg or provide a priming event for a memory response to the licensed recombinant protein vaccine when a low dose was used (0.25 μg, two doses; Ref. 28). This is consistent with our observations in pig-tailed macaques, where only one of three animals receiving repetitive doses of pCEA/HBsAg by gene gun developed HBsAg antibodies, whereas all three animals receiving the DNA vaccine i.m. seroconverted (15). However, a subsequent study in humans using particle-mediated delivery of higher doses of DNA resulted in seroconversion of all 12 healthy volunteers (29).

In the present study, HBsAg antibodies were induced within 9 weeks of primary immunization, a constraint imposed by vaccination of patients with limited life expectancy because of metastatic colorectal carcinoma. We have reported previously that only one of three pig-tailed macaques receiving repetitive 1-mg doses of pCEA/HBsAg by i.m. injection developed HBsAg antibodies within 19 weeks after primary immunization, whereas all three seroconverted to protective levels of antibody by week 28 (15). Thus, a more protracted vaccination schedule may increase the frequency of seroconversion to protective levels of HBsAg antibodies, particularly when lower doses of DNA are used. Indeed, in the most promising clinical study to date using multiple DNA immunizations by PowderJect, 11 of 12 patients required >12 weeks before protective levels of anti-HbsAg antibodies were achieved (29). Another factor which may have diminished the frequency of anti-HBsAg seroconversion in the present study was the use of a dual expression plasmid with two separate CVM promoter/enhancers. Promoter interference may have reduced the expression level of each antigen as compared with the use of two separate plasmids (30). A lack of detectable *in vitro* lymphoproliferative responses to HBsAg in the present study was not unexpected, because such responses are weak or absent after viral infection or repetitive immunization with the commercial HBsAg protein vaccine (31–33). Nevertheless, the present study has demonstrated the efficacy of i.m. DNA vaccination in humans regarding induction of protective serological responses to HBsAg.

The third aim of the study was to evaluate any CEA-specific immune responses or antitumor effects induced by the vaccine. CEA-specific antibody responses were not observed. However, 4 of 17 patients developed lymphoproliferative responses to CEA after vaccination. The low frequency of responses may have resulted in part from the compromised immune status of these patients with advanced colorectal carcinoma. This premise is supported by the observation that proliferative responses to a recall antigen, tetanus toxoid, diminished significantly in these patients during their period of study participation. We have reported previously a similar decrease in

Table 2 Specificity of the lymphoproliferative response of selected patients immunized with pCEA/HBsAg

Patient	Weeks after primary immunization	Media	CEA	gp160[a]	BSA	PHA
6	6	40 ± 4[b]	1,300 ± 200	110 ± 70	42 ± 8	21,000 ± 1,000
7	9	30 ± 6	110 ± 10	28 ± 10	28 ± 7	38,000 ± 7,000
10	6	32 ± 8	2,500 ± 700	17 ± 2	17 ± 5	51,000 ± 10,000
10	9	55 ± 8	420 ± 20	49 ± 20	30 ± 7	65,000 ± 4,000
16	6	84 ± 10	290 ± 60	106 ± 10	68 ± 30	17,000 ± 700

[a] Baculovirus recombinant HIV gp160 protein.
[b] Values are mean cpm of quadruplicate wells ± SE.

recall antigen responsiveness in patients with metastatic adenocarcinoma (34).

An increasing body of literature has been reported, exploring different vaccination strategies targeting CEA (reviewed in Ref. 35). Our group has recently reported induction of CEA-specific autoantibodies by a recombinant vaccinia virus encoding CEA (rV-CEA; Ref. 23). Schlom's group has also described the derivation of CEA-specific cytolytic T-cell lines after rV-CEA immunization (36). However, lymphoproliferative responses to CEA were not observed in either trial (34, 36). T-cell responses have been reported in subsequent pox-based CEA immunization strategies that included costimulatory or immunostimulatory molecules (35). CEA-specific antibody and lymphoproliferative responses have been reported after vaccination with an anti-idiotype monoclonal antibody mimicking a portion of the CEA molecule in patients with colorectal carcinoma (37). Finally, dendritic cell-based vaccines have also shown T-cell and clinical responses (35). Collectively, these CEA immunization strategies demonstrate that breaking immunological tolerance to this molecule is feasible, generally safe, and shows some clinical promise.

The incidence of CEA-specific lymphoproliferative responses was low in this pilot Phase I trial. Also, these immune responses were not associated with objective tumor regression or sustained declines in circulating CEA, and there was not a good correlation between positive lymphoproliferation and stable disease. This is consistent with the finding of others demonstrating a lack of correlation between *in vitro* assays and clinical outcome (38–40). Several factors may have contributed to this suboptimal immune response. As discussed above, an extended immunization period may favor a more robust immune response. Alternatively, prime-boost immunization strategies combining different vaccines may further enhance immune responses (41). Thus, additional Phase I/II trials of CEA polynucleotide immunization examining augmentation strategies such as cytokine or costimulatory molecule cDNA codelivery with or without priming by rV-CEA or recombinant canarypox are warranted in an effort to increase the frequency and magnitude of CEA-specific T-cell responses before Phase III trials. Also, given the favorable safety profile of DNA vaccines thus far, future trials of CEA immunization should focus perhaps on patients with less advanced disease.

This and other first-generation clinical trials have indicated that DNA vaccination is safe and well tolerated. Furthermore, the present study has unequivocally demonstrated induction of primary immune responses to HBsAg in the majority of patients receiving repetitive doses. This study, together with the other Phase I trials of a malarial and hepatitis B DNA vaccines (11, 29), provides the most encouraging human immune response data to date in support of DNA vaccination. The next decade promises to provide much insight into the clinical utility of this novel vaccine technology in the fields of infectious disease and cancer.

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Cytokine Enhancement of DNA Immunization Leads to Effective Treatment of Established Pulmonary Metastases

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Abstract

DNA immunization can result in the induction of Ag-specific cellular and humoral immune responses and in protective immunity in several Ag systems. To evaluate the utility of DNA-based immunization as a potential cancer treatment strategy, we employed an experimental murine tumor, CT26, expressing the model tumor-associated Ag, β-galactosidase (β-gal), designated CT26.CL25. A plasmid expressing β-gal (pCMV/β-gal) administered by particle-mediated gene delivery to the epidermis using a hand-held, helium-driven "gene gun" induced β-gal-specific Ab and lytic responses. Immunization with this construct prevented the growth of pulmonary metastatic tumor, and the adoptive transfer of splenocytes generated by pCMV/β-gal in vivo immunization and cultured in vitro with the $\beta\text{-gal}_{876–884}$ immunodominant peptide reduced the number of established pulmonary nodules. DNA immunization alone had little or no impact on the growth of established lung metastases. To enhance the function of DNA immunization for active immunotherapy, a panel of cytokines was added as adjuvants following DNA administration. Significant reduction in the number of established metastases was observed when human rIL-2, mouse rIL-6, human rIL-7, or mouse rIL-12 were given after DNA inoculation; mouse rIL-12 as an adjuvant had the most profound effect. These findings suggest that the cytokines involved in the activation and expansion of lymphocyte populations may improve the therapeutic effects of DNA immunization. Given the ease with which plasmid DNA can be prepared to high purity for safe use in humans with infectious diseases and cancers, DNA immunization administered together with cytokine adjuvant may be an attractive alternative to recombinant viral vaccines.

The induction of antitumor immunity, in part, involves CTL responses (1-3). The recent cloning of genes encoding tumor-associated Ag (TAA)3 recognized by CTL makes it possible to design Ag-specific recombinant viral and nonviral vectors that allow for control over parameters such as the quantity and kinetics of expression, the intracellular compartment into which the TAA are expressed, and the tissues or cell types that are used to express TAA in vivo (4-9). For example, several viruses including recombinant vaccinia virus, fowlpox virus, and adenovirus encoding model TAAs have been shown to express Ags within the cytoplasm of infected cells, resulting in the induction of tumor immunity (10-12,12a). However, immunization with live attenuated or recombinant viruses, although potent, may also pose safety problems due to their infectious nature (13). In addition, immune responses against heterologous protein may be reduced when the host has had previous exposure to the virus such as has been observed with vaccinia virus (14-16).

DNA-based immunization is an attractive nonviral alternative for cancer immunotherapy. DNA vaccination is accomplished by the expression of inoculated bacterial plasmid DNA encoding the foreign gene of interest accompanied by a mammalian promoter/enhancer, and other sequences such as Kozak's consensus sequence and leader sequences that enable the gene

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to be expressed within mammalian cells utilizing host machinery (17-19). In this report, a hand-held helium-powered device was used to achieve the direct intracellular delivery of DNA-coated gold particles to the epidermis. Following delivery, the DNA redissolves in the aqueous environment of the cytoplasm or nucleus and is then available for expression (20-25). Alternatively, skeletal muscle cells have demonstrated the ability to take up and express DNA for approximately the lifetime of the mouse without any specific delivery system (17,18, 26-28). However, epidermal gene gun immunization of DNA may be more efficient than intramuscular immunization at eliciting similar immune responses (22,25).

Both DNA-based approaches have been shown to successfully induce both humoral and cellular immunity in many Ag systems (22-25,29-34). Gene gun delivery of DNA encoding human growth hormone (hGH) resulted in specific Ab responses that correlated with particle delivery to the mouse epidermis (23,29). Intramuscular inoculation of plasmid DNA encoding the influenza A viral nucleoprotein could induce protective humoral and cellular immunity (30). Protective immunity after DNA-based immunization has also been observed using gp160 and rev proteins from HIV I, and H1 Ag from influenza, circumsporozoite protein from malaria, and nucleoprotein from lymphocytic choriomeningitis virus (22,31-34). In cancer immunotherapy, plasmid constructs encoding either the full length cDNA for carcinoembryonic Ag (CEA) or HIV-1 envelope protein, gp160, have been shown to protect mice from subsequent challenge with syngeneic tumors expressing these model Ags (35-37).

To the best of our knowledge, no group has shown therapeutic activity against established tumors using either "naked" DNA injection or gene gun immunization. In this study, we used the murine colon adenocarcinoma (CT26.WT) transfected with the gene for the model TAA, β-gal (CT26.CL25) (12), to test the antitumor potential of a plasmid DNA-based vaccine using a gene gun delivery system. Here, we show that DNA-based vaccines enhanced with the systemic administration of rhIL-2, rmIL-6, rhIL-7, or rmIL-12 may have utility in the active immunotherapy of cancer.

Materials and Methods

Tumor cell lines and animals

CT26.WT is a clone of the *N*-nitroso-*N*-methylurethan-induced BALB/c (H-2^d) undifferentiated colon carcinoma (38). Following transduction with a retrovirus encoding the *LacZ* gene, CT26.WT was subcloned to generate the β-gal-expressing cell line CT26.CL25 (12). All tumor cell lines were grown and maintained in complete medium (CM) containing RPMI 1640, 10% heat-inactivated FCS (both from Biofluids, Rockville, MD), 0.03% fresh L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from National Institutes of Health, Media Unit, Bethesda, MD), and 50 μg/ml gentamicin sulfate. CT26.CL25 was grown in the presence of 400 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Female BALB/c mice, 6 to 10 wk old, were obtained from the Animal Production Colonies, Frederick Cancer Research Facility. National Institutes of Health (Frederick, MD).

Plasmid preparations

A plasmid encoding the *Escherichia coli Lac2* gene under the control of the human CMV intermediate-early promoter, designated pCMV/β-gal, was kindly provided by J. Haynes (Agracetus, Middleton, WI). Plasmids expressing either human growth hormone (pCMV/ hGH), provided by J. Haynes, or the nucleoprotein from influenza A (A/pR/8/34) (pCMV/NP) under the control of the CMV intermediate-early promoter were used as control vectors in this study. Constructs were transformed into *E. coli* DH5 α-competent cells (Life Technologies, Inc.) and grown in L-broth with 100 μg/ml ampicillin as described (39). Closed circular plasmid

DNA was isolated using Wizard Maxipreps DNA purification kits (Promega Corp., Madison, WI). The 260/280 ratios ranged from 1.8 to 2.0.

Peptides

The synthetic peptide TPHPARIGL, representing the naturally processed H-2 L^d-restricted epitope spanning 876–884 of β-gal (40) and the influenza $NP_{147-155}$ peptide, TYQRTRALV, presented by H-$2K^d$, were synthesized by Peptide Technologies (Washington, DC) to a purity of greater than 99% as assessed by HPLC and amino acid analysis.

Gene gun delivery of DNA

Plasmid DNA was affixed to gold particles by adding 10 to 50 mg of 0.95-μm-diameter gold powder (kindly provided by Agracetus, Middleton, WI) to a 1.5-ml centrifuge tube containing 100 μl of 0.1 M spermidine (Sigma Chemical Co., St. Louis, MO). Plasmid DNA and gold were coprecipitated by the addition of 200 μl of 2.5 M $CaCl_2$, during vortex mixing as previously described (24). After settling for 10 min, the precipitate was washed with absolute ethanol to remove H_2O and resuspended at either 3.5 mg gold/ml or 7.0 mg/ml of ethanol, which would result in 0.25 mg or 0.5 mg of gold particles per shot, respectively. While the amount of gold per shot remained constant, the total amount of DNA per shot ranged from 0.001 to 1.0 μg per shot. Animals were anesthetized with 200 μl of a 9% solution of sodium pentobarbital while abdominal areas were shaved. DNA-coated gold particles were delivered into abdominal epidermis using the hand-held, helium-driven Accell gene delivery system (kindly provided by Agracetus). Each animal received 2 to 10 nonoverlapping deliveries per immunization (as designated below), at a pressure of 400 psi of helium.

Enzyme-Linked Immunosorbent Assay

BALB/c mice were immunized two times at 2-wk intervals with 0.001 to 1.0 μg of either pCMVIP/β-gal or pCMV/NP using the gene gun. Serum samples were collected 2 wk following the second immunization and analyzed for the presence of anti-β-gal Abs by ELISA. Specifically, microtiter plates were dried overnight at 37°C in a nonhumidified incubator with 200 ng/well/50 μl of either purified β-gal or control Ag, ovalbumin (both obtained from Sigma Chemical Co., St. Louis, MO). Incubation of 100 μl of 5% BSA in PBS on each well for 1 h to prevent nonspecific Ab binding was followed by a second 1-h incubation with 50 μl of fivefold dilutions (starting at 1:100) of test sera or control anti-β-gal murine mAb (starting at 100 ng/50 μl). After washing with 1% BSA in PBS, horseradish peroxidase-conjugated sheep anti-mouse IgG $F(ab')_2$, fragments (1:3000) (Amersham International, Amersham, UK) were added for 1 h at 37°C to detect Abs immobilized on the wells. The resulting complex was detected by the chromogen, *o*-phenylenediazamine (Sigma Chemical Co.). Absorbance was read on a Titertek Multiskan Plus reader (Flow Laboratories, McLean, VA) using a 490-nm pore diameter filter. Concentrations of β-gal-specific Ab in serum samples were estimated from the mAb standard curve and expressed as μg/ml.

Effector cells

Primary lymphocyte populations were generated by immunization with different amounts of purified pCMV/β-gal or pCMV/NP. Secondary in vitro effector populations were generated by harvesting spleens of mice 14 days after immunization and culturing single cell suspensions of splenocytes in T-75 flasks (Nunc, Roskilde, Denmark) at a density of 3.0×10^6 cells/ml with 1 μg/ml antigenic peptide at a total volume of 30 ml of CM containing 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (both from Biofluids), and 5×10^{-5} M 2-ME (Life Technologies, Inc., Rockville, MD) in the absence of IL-2. Six days later splenocytes were

harvested and washed in CM before testing in a ^{51}Cr release assay or for transfer to tumor-bearing animals.

^{51}Cr release assay

Six-hour ^{51}Cr release assays were performed as previously described (41). Briefly, 2×10^6 target cells were incubated in 0.2 ml of CM labeled with 200 μCi of $Na^{51}CrO_4$ for 90 min. Peptide-pulsed CT26.WT were incubated with 1 μg/ml (approximately 1 μM) antigenic peptide during labeling as previously described (42). Target cells were then mixed with effector cells for 6 h at 37°C at the effector to target ratios indicated. The amount of ^{51}Cr released was determined by gamma counting and the percentage of specificlysis was calculated as follows.

$$\% \text{ specific lysis} = [(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})] \times 100.$$

In vivo studies

For in vivo prevention studies, mice were immunized with different amounts of pCMV/β-gal or either control pCMV/hGH or pCMV/NP. Fourteen days later, mice were challenged i.v. with 2×10^5 tumor cells (43). Mice were killed on day 17 and randomized before counting lung metastases in a blind fashion as previously described (43).

Adoptive immunotherapy experiments

BALB/c mice were injected i.v. with 2×10^5 CT26.WT (β-gal$^-$) or CT26.CL25 (β-gal$^+$) cultured tumor cells in 0.5 ml of HBSS to induce pulmonary metastases. On day 3, tumor-bearing mice were treated with an i.v. injection of various effect or cells at 5×10^6 cells/dose. Specifically, for the generation of effector cells, mice were immunized with 1 μg of pCMV/β-gal or pCMV/NP. Each mouse received two 0.25-mg shots of gold loaded with 0.5 μg of DNA. Two weeks later, splenocytes were harvested and cultured for 6 days in CM plus 5×10^{-5} M 2-ME, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate with 1 μg/ml of either β-gal$1_{876-884}$ or $NP_{147-155}$ synthetic peptide. On day 17 after tumor injection, mice were killed and pulmonary metastases were enumerated in a coded, blind fashion. When metastases exceeded 250, they were deemed too numerous to count.

In vivo treatment studies

BALB/c mice were challenged with 5×10^5 CT26.WT or CT26.CL25 tumor cells i.v. to establish pulmonary metastases. On day 2, mice were immunized with 10 μg of pCMV/β-gal, pCMV/NP DNA, or no DNA. Each mouse received 10 nonoverlapping shots of gold (0.5 mg each) delivering l μg of DNA. On day 12, the mice were killed and metastatic lung nodules were enumerated in a randomized, blind manner. As a positive control, a group of mice was included that received a recombinant vaccinia virus encoding the β-gal (VJS6), plus the exogenous administration of the cytokine, IL-2 (15,000 U, twice daily (BID) for 5 days) as previously reported (44).

Intraperitoneal treatments of various cytokines began 18 to 24 h following DNA administration and continued daily for 3 to 7 days depending on the cytokine. Specifically, one group of mice received 15,000 Cetus U of rhIL-2, BID, for 5 days (Chiron Corp., Emeryville, CA) (44). A second group of mice received treatments of 0.5 μg of rmIL-6, BID, for 3 days (Peprotech, Inc., Rocky Hill, NJ). A third group received 5 μg of rhIL-7, BID, for 7days (Peprotech). A fourth received 1.0 μg of rmIL-12 once daily (QD) for 5 days (Genetics Institute, Boston, MA). In a screening experiment of many cytokines (data not shown), rmIL-4 (5 μg, BID, 7 days), rmIL-10 (1.0 μg, QD, 7 days), and GM-CSF (1.0 μg, QD, 5 days) were also assayed for adjuvant activity (Peprotech). These parameters were determined from previous reports demonstrating antitumor immune responses from their exogenous administration (45-50). These doses of

cytokine alone were shown to have little or no effects on the growth of CT26.WT of CT26.CL25.[4]

Statistical analysis

Because lungs that contained >250 pulmonary metastases were deemed too numerous to count, the data do not follow a normal distribution. Thus, statistical evaluation of the data was performed using the nonparametric two-tailed Kruskal-Wallis test.

Results

DNA-based vaccine elicits Ag-specific humoral and cellular immunity

To study the induction of Ag-specific humoral immunity using a DNA-based vaccine, mice were immunized with a plasmid cDNA encoding the model TAA β-gal (pCMV/β-gal). Mice immunized and boosted with gold particles coated with as little as 0.01 μg of pCMV/β-gal developed β-gal-specific Ab responses (Fig. 1). In contrast, gold particles coated with 1.0 μg of the control plasmid pCMV/NP failed to elicit β-gal-specific Ab. No reactivity was observed against a control Ag, ovalbumin, confirming the specificity of the humoral immune response (data not shown).

To test whether a CTL response could be elicited against β-gal expressed by DNA immunization, BALB/c mice were inoculated once with varying quantities of pCMV β-gal or control vector, pCMV/NP. Splenocytes from mice that were injected with as little as 0.1 μg of pCMV/β-gal and then restimulated in vitro with the β-gal$_{876–884}$ peptide lysed the β-gal-transfected tumor, CT26.CL25 (Fig. 2). CT26.WT pulsed with the immunodominant peptide β-gal$_{876–884}$ was also recognized by the pCMV/β-gal-immune splenocytes generated with as little as 0.01 μg of DNA. Pulsed cells in this case appeared to be more sensitive to the lytic cells than the transfected cells, CT26.CL25. Unpulsed CT26.WT was not significantly lysed, demonstrating the specificity of the lytic response. Splenocytes derived from mice immunized with pCMV/NP and cultured in vitro in an identical manner as described above failed to lyse CT26.WT. CT26.WT pulsed with the β-gal$_{876–884}$ or CT26.CL25. Together, these data indicated that DNA administered with the gene gun was able to elicit specific humoral and cellular immunity against the model tumor Ag, β-gal.

Prophylactic DNA vaccine protects mice from intravenous tumor challenge

To determine whether humoral and cellular responses observed in vitro correlated with in vivo antitumor activity, mice were immunized with the DNA vaccine and assayed for the growth of a subsequent i.v. tumor challenge. Only the mice that received pCMV/β-gal showed significant responses compared with the mice that were inoculated with control DNA (Fig. 3). In pooled results from three experiments, almost complete protection was observed with 1.0 μg of pCMV/β-gal; 19 of 20 pairs of lungs from immunized mice were devoid of any detectable tumor. At a 10-fold lower dose, 16 of 20 pairs of lungs were completely free of disease. With a dose of 0.01 μg of DNA immunized, the protective effects began to wane, with only 6 of 20 mice disease free. This correlated with decreasing amounts of Ab and CTL activity observed in the mice that received 0.01 μg of DNA (Figs. 1 and 2). In addition, mice immunized with pCMV/β-gal and then challenged with β-gal$^-$ CT26.WT were not protected from tumor growth (Fig. 3). Therefore, these data suggest that the protection from tumor following gene gun inoculation of DNA encoding a TAA is Ag specific both at the level of the immunizing plasmid and at the level of Ag expression by tumor.

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[4]Rao, J . B., V. Bronte, K. R. Irvine, M. W. Carroll, S. A. Rosenberg, and N. P. Restifo. *Submitted for publication.*

Adoptive transfer of splenocytes generated using DNA vaccine mediate reduction of established pulmonary metastases

To ascertain whether the effector cells elicited by gene gun immunization were active in in vivo adoptive immunotherapy, we used a 3-day lung metastasis model (43). In these experiments, BALB/c mice were given i.v. injections with either CT26.CL25 (β-gal$^+$) or CT26.WT (β-gal$^-$) tumor cells. On day 3, tumor-bearing mice were treated with splenocytes from donor mice that had received gene gun immunization with either pCMV/β-gal or pCMV/NP, and then subsequent ex vivo incubation with either β-gal$_{876–884}$ or control NP$_{147–155}$ peptide.

Effector cells generated from mice immunized with pCMV/β-gal and cultured with β-gal$_{876–884}$ peptide completely cleared the lungs of mice bearing 3-day-old pulmonary metastases (Fig. 4). By contrast, lungs from CT26.CL25-bearing mice that received either no splenocytes, splenocytes from pCMV/β-gal-immunized mice stimulated with the irrelevant synthetic peptide (NP$_{147–155}$), or splenocytes from pCMV/NP-immunized mice stimulated with the β-gal$_{876–884}$ peptide, contained >250 metastases. In addition, the splenocytes induced by pCMV/β-gal and stimulated with β-gal$_{876–884}$ peptide were not effective at eliminating metastases in mice bearing the Ag-negative tumor, CT26.WT (Fig. 4), demonstrating the in vivo specificity for the β-gal Ag. Similar results were observed in a repeat experiment. Thus, the induction of immune splenocytes for adoptive transfer was Ag specific, and the therapeutic activity of these cells was specific for Ag expression by the tumor. The potential therapeutic implications of this approach are clear, since this strategy involved not just the prevention of disease, but the treatment of established metastases.

Gene gun immunization of DNA alone induces little to no active specific immunotherapy of established pulmonary metastases

Mice bearing 2-day established pulmonary metastases were immunized with Pcmv/β-gal to evaluate the ability of gene gun immunization of DNA to generate active specific therapeutic responses. Initial experiments were carried out in mice given 5×10^5 tumor cells as reported previously (44). Seven independent attempts to use gene gun immunization alone to treat established pulmonary metastases have been made with only one experiment demonstrating a reduction in tumor growth. When the number of tumor cells given was reduced to 10^5, significant reduction in the number of metastases or mouse survival was observed in groups receiving β-gal-specific gene gun immunization in two of three experiments performed (data not shown).

Cytokine administration following DNA vaccine leads to treatment of established pulmonary metastases

Several cytokines, known to have different immune-regulating effects, have been reported to have antitumor activity as single agent therapy (46-48,51) as well as adjuvant to standard therapy (46,52-54). For example, rhIL-2, rmIL-6, and rhIL-7 have been reported to activate or proliferate Ag-specific CTL populations (47,55). GM-CSF promotes the differentiation of hematopoietic precursors to dendritic cells that function to present Ag to prime naive lymphocytes (56). rmIL-12, rmIL-4, and rmIL-10 have been shown to direct Th populations to different Th1 or Th2 phenotypes, resulting in shifts to either humoral or cell-mediated responses, respectively (57-60). A screening assay was performed to determine whether various cytokines could act as adjuvants to DNA vaccination to induce active specific immunotherapy. In these experiments, tumor-bearing mice immunized on day 2 with pCMV/βgal or unimmunized were treated 18 to 24 h later with either rhIL-2, rmIL-4, rmIL-6, rhIL-7, rmIL-10, rmIL-12, or rmGM-CSF administered exogenously. From this screening assay, only rhIL-2, rmIL-6, rhIL-7, and rmIL-12 were found to specifically induce Ag-specific active immunotherapy (the entire screen is not shown).

Figure 5 illustrates the average number of pulmonary metastases on day 12 following tumor challenge for those mice that received the DNA vaccine followed by either rhIL-2, rmIL-6, or rhIL-7 administration. Figure 5 represents the pooled averages of two separate experiments done with the same protocol. Mice that were injected with pCMV/β-gal alone demonstrated no significant reduction in the number of metastases (215) compared with control non-immunized mice (>250). The groups of mice that received pCMV/β-gal administration plus individual cytokines rhIL-2, rmIL-6, or rhIL-7 all demonstrated a significant reduction in number of metastases compared with either cytokine alone (all >250, $p_2 = 0.001$, $p_2 = 0.003$, and $p_2 = 0.0002$, respectively), or β-gal alone (>250, $p_2 = 0.003$, $p_2 = 0.0003$, and $p_2 = 0.001$, respectively).

In an extensive experiment evaluating IL-12, treatment with pCMV/β-gal alone showed a slight but significant reduction in the number of pulmonary metastases compared with the group that received pCMV/NP (Fig. 6, 151 compared with 239, $p_2 = 0.016$). This result was only observed one time out of seven experiments that were performed. rmIL-12 alone demonstrated a modest reduction in the tumor burden with an average of 177 ± 45 metastatic nodules compared with >250 in the untreated group. A dramatic reduction was observed with the combination of rmIL-12 and pCMV/β-gal (an average of 8 ± 2.5 metastases) (Fig. 6). This was significant in comparison to groups that received pCMV/β-gal alone ($p_2 = 0.012$), rmIL-12 alone ($p_2 = 0.002$), and pCMV/NP plus rmIL-12 ($p_2 = 0.008$). pCMV/β-gal resulted in a significant reduction in tumor burden even with lower doses of rmIL-12. Doses of 0.3 and 0.1 μg of rmIL-12 were also able to diminish the tumor burden to 17 ± 3.8 and 32 ± 9.2 metastases, respectively. These data were significant compared with controls, pCMV/β-gal alone ($p_2 = 0.036$ and $p_2 = 0.036$, respectively) and rmIL-12 alone ($p_2 = 0.002$ and $p_2 = 0.004$, respectively). Therefore, when rmIL-12 is administered as an adjuvant with pCMV/β-gal DNA, lower doses of rmIL-12 can be given and therapeutic effects are still observed. This kind of active treatment experiment using IL-12 as an adjuvant to DNA immunization has been repeated three times with similar findings. Together, these studies show that cytokines rhIL-2, rmIL-6, rhIL-7, and rmIL- 12 given individually but in combination with pCMV/β-gal can lead to Ag-specific reduction in tumor burden.

Discussion

To our knowledge, this study represents the first report of the use of gene gun immunization for the prevention and treatment of an experimental cancer. Plasmid DNA delivered by particle-mediated delivery induced potent humoral and CTL lytic immune responses that presumably aided in protective immunity from tumor challenge. Despite these potent responses, active immunity of established tumor with the DNA vaccination alone failed to have an impact on tumor burden. Therapeutic responses in tumor-bearing animals could be improved; however, only when particular cytokines (rhIL-2, rmIL-6, rhIL-7, and rmIL-12) were given following DNA administration.

DNA immunization resulted in potent Ag-specific Ab and CTL responses (Figs. 1 and 2). These findings were consistent with other reports of the use of DNA-based immunizations using either particle-mediated delivery to the epidermis or direct injection intramuscularly (17,18, 20,23-27). We opted for gene gun delivery of DNA based on studies that compared different routes of immunization. Fynan et al. demonstrated that the particle bombardment method required 250 to 2500 times less DNA than saline intramuscular inoculations to induce protective immunity from viral challenge (22). Similarly, Pertmer et al. found that direct inoculation of DNA intradermally or intramuscularly required 5000-fold more DNA to achieve comparable results (25). In our studies, as little as 0.1 to 0.01 μg of DNA was observed to induce potent and consistent CTL and Ab responses. In studies not shown, at least 50 μg administered intramuscularly would induce Ab and CTL responses, but inconsistently.

However, we did not utilize muscle regeneration reagents such as bupivicaine (31), which have been reported to facilitate the uptake and transcription of the introduced DNA and therefore improve the immune responses observed.

Several other groups have described various direct injection DNA-based immunization protocols resulting in the prevention of not only tumor but also viral and nonviral diseases (22,31-37). Recently, Conry et al. have developed a plasmid encoding full length cDNA for human CEA under the transcriptional control of the CMV early promoter/enhancer, which functioned to elicit CEA-specific humoral and cellular proliferation responses as well as protection against a syngeneic, CEA-expressing colon carcinoma cell line (35,36). Wang et al. have also reported that the use of a plasmid encoding the envelope region of HIV (gp160) can induce long-lasting protection in mice from tumors expressing that gene (37).

In our study, DNA-based immunization was used to prime T lymphocytes reactive with the TAA before ex vivo expansion and adoptive immunotherapy, a strategy that was ineffective in the absence of in vivo priming. This represents a novel use of the gene gun approach and the strategy of using DNA as an immunogen for the treatment of established tumor. The poor performance of the DNA-based approach to treat established tumor in an active immunotherapy model suggested that the immune responses demonstrated in Figures 1and 2 were either not rapid enough in their kinetics or were below a threshold required to treat tumor. Unlike poxvirus-based vectors, DNA vaccines may not produce the quality of the antigenic protein required to elicit therapeutic antitumor responses in an active treatment regimen. The level of Ag production or persistence from the vaccine as well as the amounts of tumor Ag expressed by the tumor may be important for treatment. Increased amounts of recombinant adenovirus expressing a TAA were critical for the observation of any treatment effects (12a) Improved vectors that augment protein expression by the addition of enhancer elements are currently being designed (61).

Active treatment could be enhanced when cytokines were utilized as adjuvants to DNA immunization. When a panel of cytokines was tested, only rhIL-2, rmIL-6, rhIL-7, and rmIL-12 were able to enhance the therapeutic responses. rhIL-2, rhIL-7, and rmIL- 12 have all been reported to stimulate or activate T cell populations as well as NK cells (45,46,57). rmIL-12 also functions to regulate immune responses by directing a Th2 to a Thl phenotype (58,60). IL-6, known as B cell-stimulatory factor-2, is a pleiotrophic cytokine that can enhance CTL function, NK activity, LAK, and TIL activity (47,62). All of these cytokines have been reported to have antitumor effects when administered as single agent therapy (46-48,63,64). However, the doses used in this study were previously shown to have little to no effect on the growth of CT26.CL25.[4] On the other hand, cytokines such as GM-CSF, IFN-γ, and IL-4, when given in the adjuvant setting, failed to mediate tumor regression. IL-4 may not have had an effect because it steers the T helper cell population toward a Th2 phenotype responsible for the enhancement of humoral responses but not of cell mediated immunity (65). IFN-γ up-regulates the production of key molecules involved in Ag processing and presentation of intracellular Ags (66) but may also have an antiproliferative effect on T cells GM-CSF was reported in earlier studies to have little to no adjuvant effect when encoded in the same recombinant virus as TAA (44). We have also observed little to no adjuvant effect when GM-CSF was administered exogenously with a rVV encoding β-gal in three of four experiments.[4] The findings in this study, that GM-CSF did not act an adjuvant, suggested that the APC are not limiting when the Ag supplied by DNA immunization.

The known functions of the cytokines that acted as adjuvants compared with those that failed to enhance therapeutic responses suggest that proliferation and activation of primed lymphocytes are important mechanisms for active treatment to occur. The use of cytokines to enhance tumor immunity has been reported previously in a number of different vaccine settings.

Sun et al. have shown that gene gun injection of cytokines rmIL-6, rhIL2, and rmTNF-α directly into the tumor mediate tumor reduction (67). Bronte et al. have shown that either exogenous administration of rhIL-2 with rVV or rhIL-2 plus Ag β-gal encoded within the vaccinia viral genome can also enhance treatment responses (44) Rao et al. have observed that other cytokines, rmIL-12 and rmIL-10, administered exogenously along with rVV-encoding β-gal can also augment therapeutic responses.[4] In addition, rhIL-2, rmIL-6, rhIL-7, and rmIL-12 have each been reported previously to have adjuvant effects in different Ag settings (46, 52-54,68).

With the particle delivery technique, several genes encoding different molecules can be coated onto each gold bead; thus each bombarded cell could potentially elaborate more than one protein. Recently, Xiang and Ertl demonstrated immune enhancement by co-injecting plasmids encoding Ag along with plasmids encoding murine cytokine genes (69). Specifically, intramuscular injection of DNA encoding GM-CSF with DNA encoding rabies glycoprotein could augment the rabies-specific Ab, as well as enhanced protection from viral challenge. In contrast, the addition of a vector containing IFN-γ did not induce increased Ab production, T cell proliferation, or protective immunity from viral challenge (69). Future studies will be directed at the development of beads that are coated with DNA encoding immune-regulating molecules as well as Ag to determine whether we can improve the antitumor immune effects.

It should be emphasized that β-gal is a molecule foreign to a mouse, and the responses elicited by the pCMV/β-gal constructs may not be the same as the responses induced by a self-Ag. However, it is intriguing that transfection of tumors with several nonself-genes, including human CEA, NP from VSV virus, and *E. coli*-derived β-gal, do not result in the rejection of the experimental tumor (11,12,70). In fact, CT26.WT and CT26.CL25 grow at equivalent rates and no secondary anti-β-gal-lytic responses have been detected in CT26.CL25-bearing mice (12). However, these nonself-Ags do bear similarities to some potential tumor Ags such as viral Ags expressed in virally induced tumors, mutated tumor suppressor genes(71), fusion proteins resulting from translocations (72), frame-shifts,[5] and loss of stop codons. Since many tumor Ags reported thus far seem to be nonmutated differentiation Ags (4-9), further studies will be aimed at evaluating effects of tolerance by either using mice transgenic for the β-gal gene or by immunization with genes encoding self-Ags within murine tumors such as the P815 mastocytoma-encoded P1A gene, and the murine homologues of the human melanoma Ags (73).

One safety issue of vaccination with nucleic acids includes the possibility of inducing an anti-DNA Ab response, which potentially could result in an autoimmune disease such as systemic lupus erythematosus. Although it is possible to generate Abs using denatured ssDNA as an immunogen, it has been shown to be difficult to induce Abs against dsDNA. In one study, primates were immunized by intramuscular injection several times, and anti-DNA Abs were not found (74). A second issue of DNA immunization is the potential integration of injected DNA into the host cell genome that may result in a transformation event. Wolff et al. addressed this point in one study in which more than 1800 recloned plasmids were analyzed for junctions between chromosomal and plasmid DNA extracted from muscle derived from mice previously immunized with DNA by direct injection (28). No evidence of DNA integration was observed. Of course, these issues will have to be addressed for particle-bombarded administration of DNA as well.

DNA-based vaccines offer several advantages over the use of recombinant viruses for immunization. Purified DNA is relatively safe compared with replication-competent viruses,

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which may result in disseminated viremia especially in immunocompromised individuals (13,14,75). Plasmid DNA can also be easily and rapidly purified in comparison to the production of live viruses, which involves time-consuming homologous recombination and plaque purification steps. The use of DNA vectors would also eliminate the problems of anamnestic responses that can eliminate recombinant viruses more rapidly, thus reducing immune responses against heterologous proteins expressed by these viral carriers (14-16). Genetic vaccines followed by cytokine treatment represent a safe alternative to recombinant viral vaccines for the evaluation of the immunotherapy of cancer.

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Abbreviations used in this paper

TAA tumor-associated Ag

β-gal β-galactosidase

NP nucleoprotein

CEA carcinoembryonic Ag

rm recombinant murine

rh recombinant human

hGH human growth hormone

CM complete medium

BID twice dally

QD once daily

GM-CSF granulocyte macrophage-CSF

rVV recombinant vaccine virus

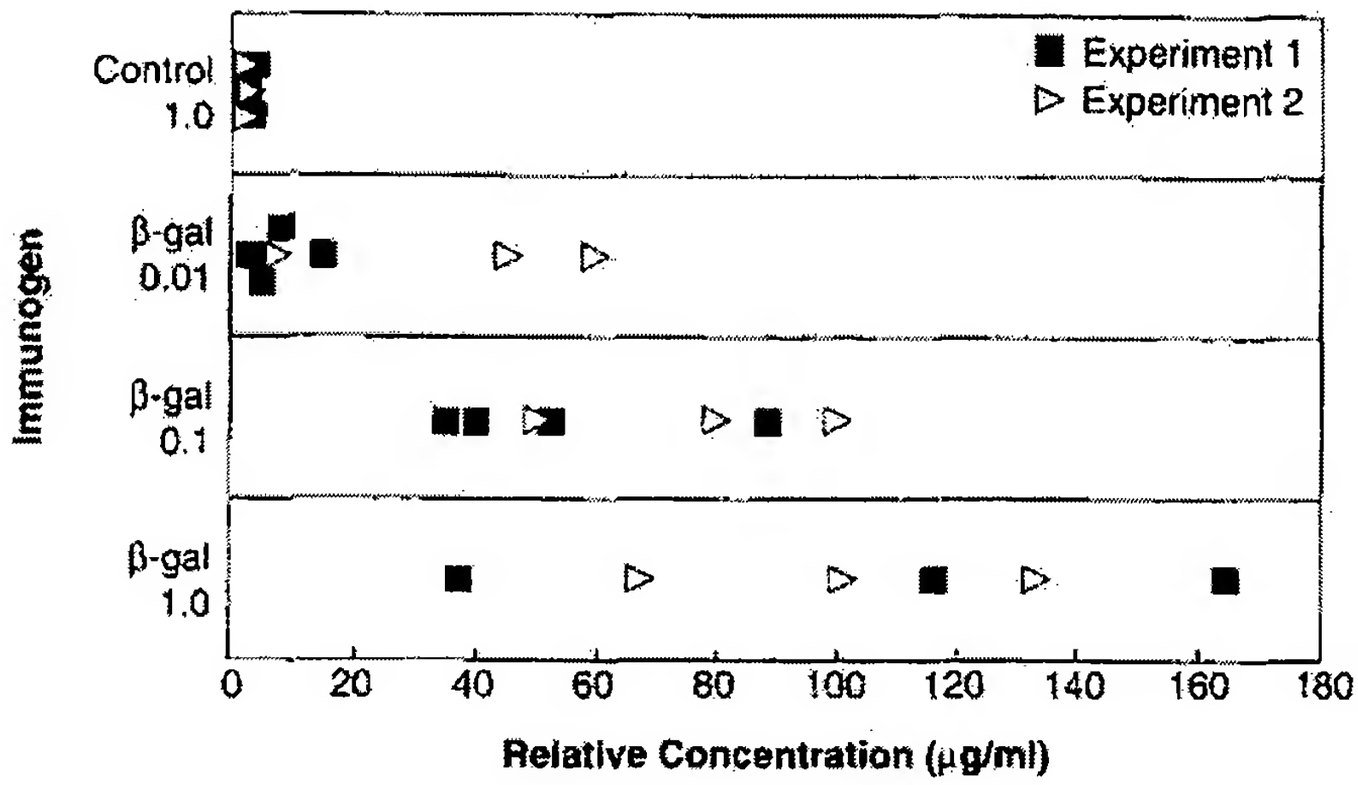

FIGURE 1.

Induction of humoral immunity elicited with gene gun vaccination of pCMV/*β*-gal. BALB/c mice (three per group) were immunized two times at 2-wk intervals into the epidermis; each immunization consisted of four shots of 0.25 mg of gold delivering a total of either 1.0 *μ*g of control pCMV/NP, or 0.01 *μ*g, 0.1 *μ*g, or 1.0 *μ*g of pCMV/*β*-gal. Fourteen days following the boost, sera were tested by ELISA for the presence of Abs against recombinant *β*-gal protein. The relative concentration of Abs reactive against *β*-gal were calculated from a standard curve of an anti-*β*-gal mAb and expressed as *μ*g/ml .

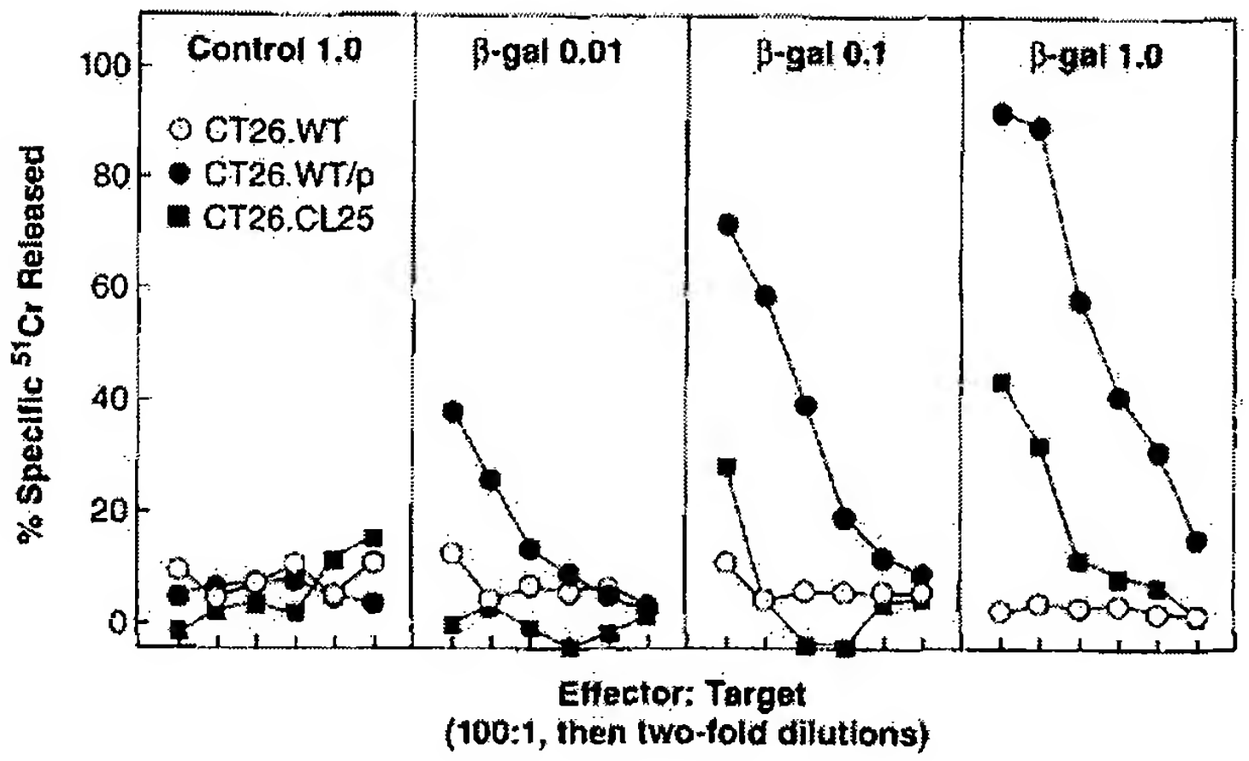

FIGURE 2.

Secondary in vitro T_{CD8+} induced by immunization with gene gun vaccination of pCMV/β-gal. BALB/c mice were immunized one time in the epidermis. Each immunization consisted of four shots of 0.25 mg of gold delivering a total of 1.0 μg pCMV/NP control DNA, 0.01 μg of pCMV/β-gal, 0.1 μg of pCMV/β-gal, or 1.0 μg of pCMV/β-gal. Fourteen days later pooled splenocytes (two mice per group) were restimulated in vitro with 1 μg of β-gal$_{876–884}$ peptide for 6 days and then assayed for specific lytic activity in a ^{51}Cr release assay against CT26.WT (β-gal^{-}), CT26.CL25 (β-gal^{+}), or CT26.WT pulsed with β-gal$_{876–884}$ peptide target cells. These experiments have been repeated two times with similar results.

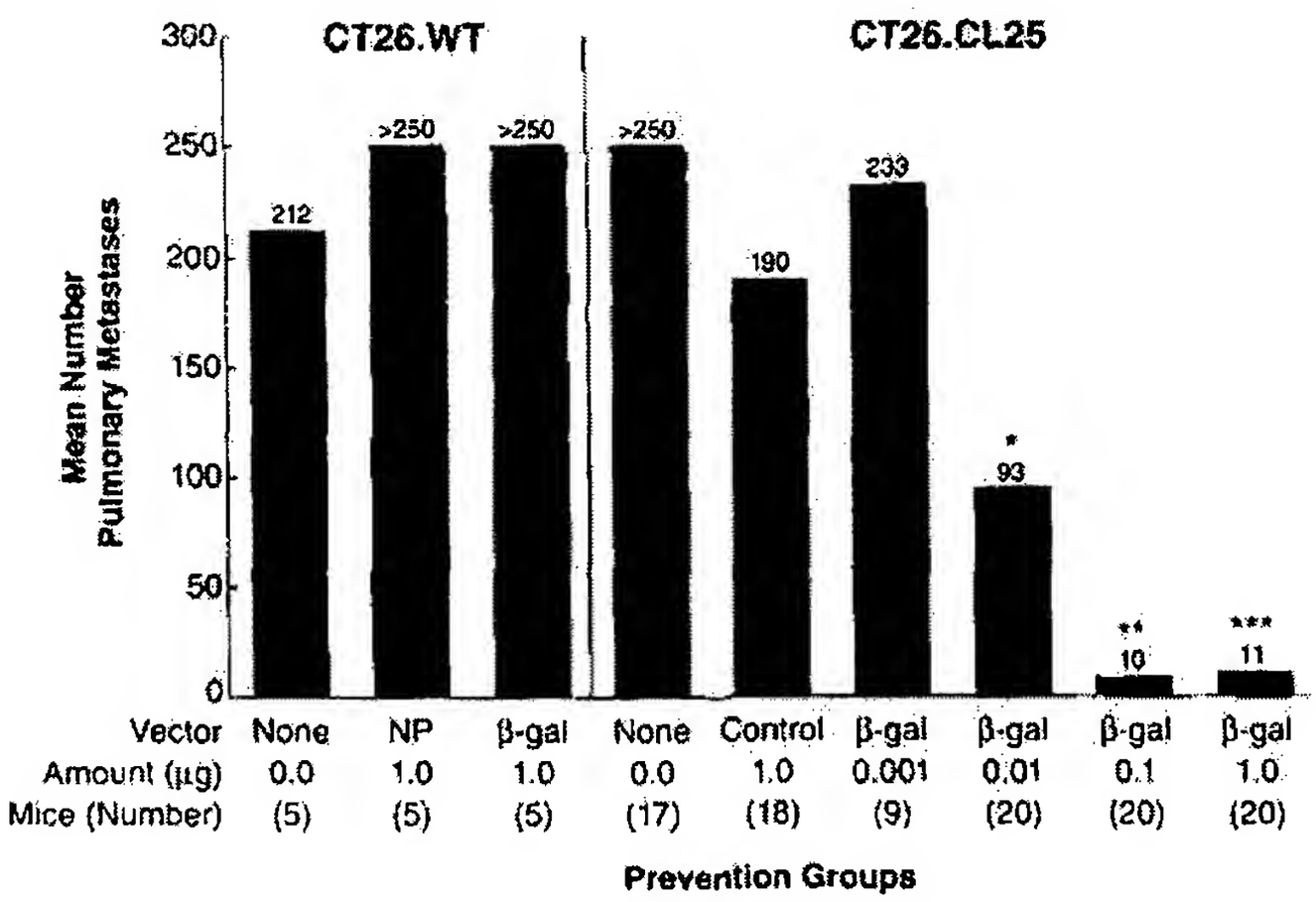

FIGURE 3.

Immunization with DNA vaccine prevents the growth of i.v. tumors. On day 0, BALB/c mice were immunized one time in the epidermis. Each immunization consisted of five shots of 0.25 mg of gold delivering 1.0, 0.10, or 0 .01 μg of pCMV/β-gal or 1.0 μg of control DNA alone. Fourteen days later, mice (5 to 10 per group) were challenged, i.v., with 2.0×10^5 CT26.CL25 (β-gal$^+$) or CT26.WT (β-gal$^-$) tumor cells. On day 17, lungs were harvested and tumor nodules were enumerated in a blind fashion. Statistical analysis was carried out using the nonparametric Kruskal-Wallis test; therefore error bars are not shown. This graph represents a summary of all the data from three separate experiments. In the first experiment, the control DNA utilized was pCMV/hGH, while in the remaining experiments, pCMV/NP was used.

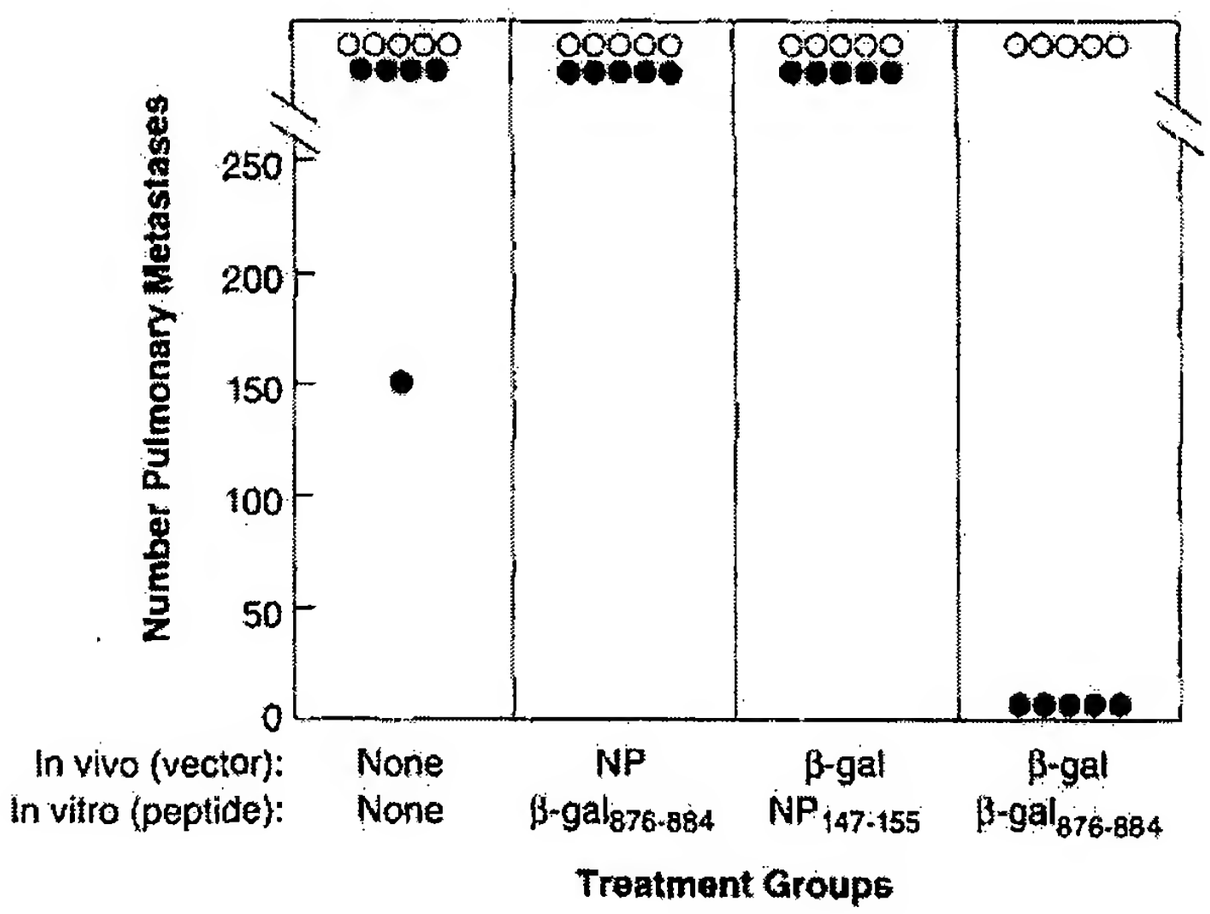

FIGURE 4.

Adoptive immunotherapy of tumor-bearing mice with immune splenocytes induced by gene gun vaccination. On day 0, CT26.WT (β-gal$^-$, ○) and CT26.CL25 β-gal$^+$, ●) tumor cell lines were each injected i.v. into BALB/c mice to create lung metastases. On day 3, tumor-bearing mice were treated with effector splenocytes from donor mice. The donor cells were generated by prior gene gun immunization with 1 μg of either pCMV/β-gal or pCMV/NP followed 14 days later by in vitro incubation for 6 days with 1 μg/ml of either β-gal$_{876–884}$ or NP$_{147–155}$ peptide. On day 17, pulmonary metastases were enumerated in a coded, blind fashion. This experiment has been repeated one time with similar findings.

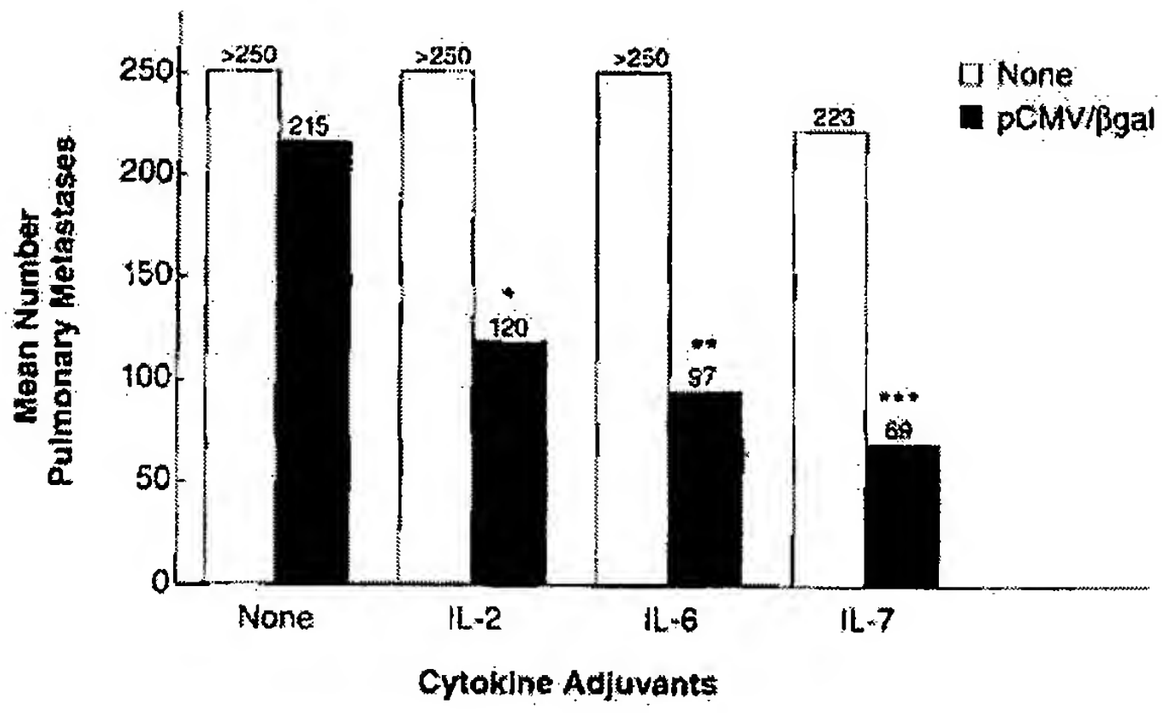

FIGURE 5.

Active immunotherapy of established pulmonary metastases with the pCMV/β-gal vaccine plus systemic administration of rhIL-2, rmIL-6, or rhIL-7. BALB/c mice were injected i.v. with 5×10^5 CT26.CL25 (βgal$^+$) tumor cells. On day 2 following tumor challenge, treated mice were immunized with 10 μg of pCMV/β-gal. Each mouse received 10 shots of 0.5 mg of gold, each shot delivering 1 μg of DNA. On day 3, mice (5 to 10 mice per group) began regimens of i.p. cytokine injections as described in *Materials and Methods*. On day 12, lungs were harvested and the pulmonary metastases were enumerated in a coded, blind fashion. Nonparametric statistical analysis was done using the Kruskal-Wallis test. The group with asterisks above were found to be statistically significant compared with either cytokine alone (*, $p_2 = 0.001$; **, $p_2 = 0.003$; ***, $p_2 = 0.0002$) or to β-gal DNA alone (*, $p_2 = 0.003$; **, $p_2 = 0.0003$; ***, $p_2 = 0.001$). The graph represents a summary of two experiments.

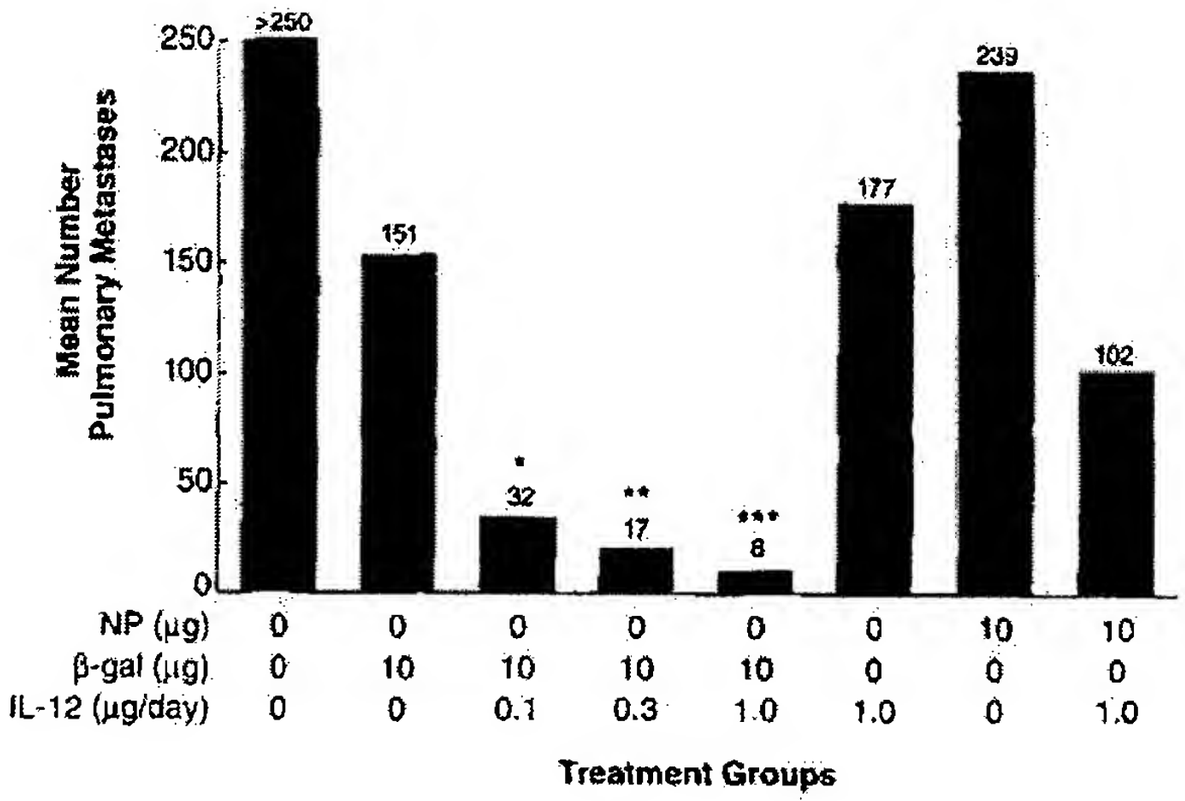

FIGURE 6.

Active immunotherapy of established pulmonary metastases with the pCMV/*β*-gal vaccine plus systemic administration of rmIL-12. BALB/c mice were injected i.v. with 5×10^5 CT26.CL25 (βgal^+) or CT26 (βgal^-) tumor cells. On day 2 following tumor challenge, treated mice were immunized with 10 μg of pCMV/*β*-gal or 10 μg of pCMV/NP. Each mouse received 10 shots of 0.5 mg of gold, each shot delivering 1 μg of DNA. On day 3, mice began regimens of i.p. rmIL-12 injections as described in *Materials and Methods*. On day 12, lungs were harvested and the pulmonary metastases were enumerated in a coded, blind fashion. Nonparametric statistical analysis was done using the Kruskal-Wallis test. The groups with asterisks were found to be statistically significant compared with either cytokine alone (*, $p_2 = 0.004$; **, $p_2 = 0.002$; ***, $p_2 = 0.002$) or *β*-gal DNA (*, $p_2 = 0.036$; **, $p_2 = 0.036$; ***, $p_2 = 0.012$) alone. The adjuvant effects of IL-12 have been observed three times, whereas the effect of *β*-gal has been observed only one time.

EXHIBIT

AE

A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors

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Anti-HER2/*neu* therapy of human HER2/*neu* expressing malignancies such as breast cancer has shown only partial success in clinical trials. To expand the clinical potential of this approach, we have genetically engineered an anti-HER2/*neu* human IgG3 fusion protein containing interleukin-2 (IL-2) fused at its carboxyl terminus. Anti-HER2/*neu* IgG3-(IL-2) retained antibody and cytokine related activity. Treatment of immunocompentent mice with this antibody fusion protein resulted in significant retardation in the subcutaneous (s.c.) growth of CT26-HER2/*neu* tumors suggesting that anti-HER2/*neu* IgG3-(IL-2) fusion protein will be useful in the treatment of HER2/*neu* expressing tumors. We also found that fusing IL-2 to human IgG3 results in a significant enhancement of the murine anti-human antibody (MAHA) response.

Keywords: Antibodies, Cytokines, Immunotherapy, Cytotoxicity, Antibody Fusion Protein

1. Introduction

The *HER2/neu* proto-oncogene (also known as *c-erbB-2*) encodes a 185 kDa transmembrane glycoprotein receptor known as HER2/*neu* or p185HER2 that has partial homology with the epidermal growth factor receptor and shares with that receptor intrinsic tyrosine kinase activity [1–3]. It consists of three domains: a cysteine-rich extracellular domain, a transmembrane domain and a short cytoplasmic domain [1–3]. Overexpression of HER2/*neu* is found in 25–30% of human breast cancer and this overexpression is an independent predictor of both relapse-free and overall survival in breast cancer patients [4–7]. Overexpression of HER2/*neu* also has prognostic significance in patients with ovarian [5], gastric [8], endometrial [9], and salivary gland cancers [10]. The increased occurrence of visceral metastasis and micrometastatic bone marrow disease in patients with HER2/*neu* overexpression has suggested a role for HER2/*neu* in metastasis [11,12].

The elevated levels of the HER2/*neu* protein in malignancies and the extracellular accessibility of this molecule make it an excellent tumor-associated antigen (TAA) for tumor specific therapeutic agents. In fact, treatment of patients with advanced breast cancer using the anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) previously known as rhuMAb HER2, directed at the extracellular domain of HER2/*neu* (ECDHER2) [13] can lead to an objective response in some patients with tumors overexpressing the HER2/*neu* oncoprotein [14,15]. However, only a subset of patients shows an objective response (5 of the 43 (11.6%)) [14,15]. Although combination of trastuzumab with chemotherapy enhances its anti-tumor activity (9 of 37 patients with no complete response (24.3%)) [16], improved therapies are still needed for the treatment of HER2/*neu* expressing tumors.

Interleukin-2 (IL-2) is a lymphokine produced by T helper cells which stimulates T cells [17–19] and natural killer (NK) cells [18] and augments antibody dependent cell-mediated cytotoxicity (ADCC) [14,15]. Although it was possible to stimulate an anti-tumor response using high doses of systemically administered

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recombinant human IL-2 (rhuIL-2) [21], the systemic administration of high-dose IL-2 had severe toxic side effects [21,22]. Targeting IL-2 to the site of a tumor with an antibody recognizing a tumor associated antigen is one means of achieving locally high concentrations of IL-2 without toxicity [23–26].

We have now expanded the family of antibody-(IL-2) fusion proteins by developing an anti-HER2/*neu* IgG3-(IL-2) fusion protein protein that may provide an effective alternative for the therapy of HER2/*neu* expressing tumors. This novel antibody fusion protein is composed by a human IgG3 with the variable region of the humanized anti-HER2/*neu* antibody, trastuzumab (Herceptin, Genentech, San Francisco, CA) [13–15] genetically fused to human IL-2. In this report we describe and discuss the strategy of construction and the properties of this novel antibody-(IL-2) fusion protein.

2. Materials and methods

Cell lines: CT26-HER2/*neu* was developed in our laboratory by transduction of CT26 cells with the cDNA encoding human HER2/*neu* [14,15]. It was cultured in Iscove's Modified Dulbecco's Medium IMDM (GIBCO, Grand Island, NY) supplemented with 5% bovine calf serum (HyClone, Logan, UT). CTLL-2, an IL-2 dependent murine T cell line (provided by Dr. William Clark, UCLA, CA) was cultured in RPMI 1640 (GIBCO) supplemented with 10% bovine calf serum and IL-2.

Mice: Female BALB/c mice 6–8 weeks of age were obtained from Taconic Farms, Inc. (Germantown, NY).

Vector construction, transfection and initial characterization of anti-human HER2/neu IgG3-(IL-2): For the construction of the heavy chain of anti-human HER2/*neu* IgG3-(IL-2), the DNA encoding the variable region of trastuzumab [13,15] was joined to a human $\gamma 3$ heavy chain containing IL-2 fused at the carboxy terminus of the C_H3 domain. It was expressed with the corresponding anti-HER2/*neu* kappa light chain in P3X63Ag8.653. Stable transfectants were selected and characterized as previously described [13,15]. The fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma Chemical, St. Louis, MO). Purity and integrity were assessed by Coomassie blue staining of proteins separated by SDS-PAGE. The ability of the fusion protein to recognize antigen was assessed by flow cytometry using CT26-HER2/*neu* cells. The ability of the fusion protein to support the growth of the IL-2 dependent cell line CTLL-2 was determined as previously described [29].

Immunotherapy: 10^6 CT26-HER2/*neu* cells in 0.15 ml HBSS were injected subcutaneously (s.c.) into the right flank of syngeneic BALB/c mice. Beginning the next day mice randomized into groups of 8 received five daily intravenous (i.v.) injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3, or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized. Blood samples were collected, serum was separated from clotted blood and stored at −20°C until assayed by ELISA.

Determination of murine anti-human HER2/neu antibodies: Sera from each treatment group were analyzed by ELISA for the presence of antibodies to human IgG3 using 96-well microtiter plates coated with 50 μl of anti-human HER2/*neu* IgG3 at a concentration of 1 μg/ml. Alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Sigma Chemical, St. Louis, MO) or rat antibodies specific for murine IgG2a, IgG2b, IgG3, IgG1 or kappa (Pharmingen, San Diego, CA) followed by alkaline phosphatase (AP)-labeled goat anti-rat IgG (Pharmingen, San Diego, CA) were used to detect bound murine antibodies. All ELISAs for comparison of titers between the experimental groups were made simultaneously in duplicate using an internal positive control curve for each plate.

Statistical analysis: Statistical analysis of the titration ELISA and anti-tumor experiment was done using a two-tailed Student's t-test. For all cases results were regarded significant if p values were $\leqslant 0.05$.

3. Results

Anti-HER2/*neu* IgG3-C_H3-(IL-2) was constructed and expressed as previously described for similar IL-2 fusion proteins [23]. Heavy and light chains of the expected size were synthesized, assembled and secreted. The fusion protein specifically bound to the human HER2/*neu* expressed on the surface of the murine cell line CT26-HER2/*neu* and was able to stimulate the proliferation of the IL-2 dependent cell line CTLL-2 with a similar proliferative response observed with equimolar IL-2 concentrations of rhuIL-2 and anti-HER2/*neu* IgG3-(IL-2) (data not shown).

To investigate in vivo anti-tumor activity, 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day mice were

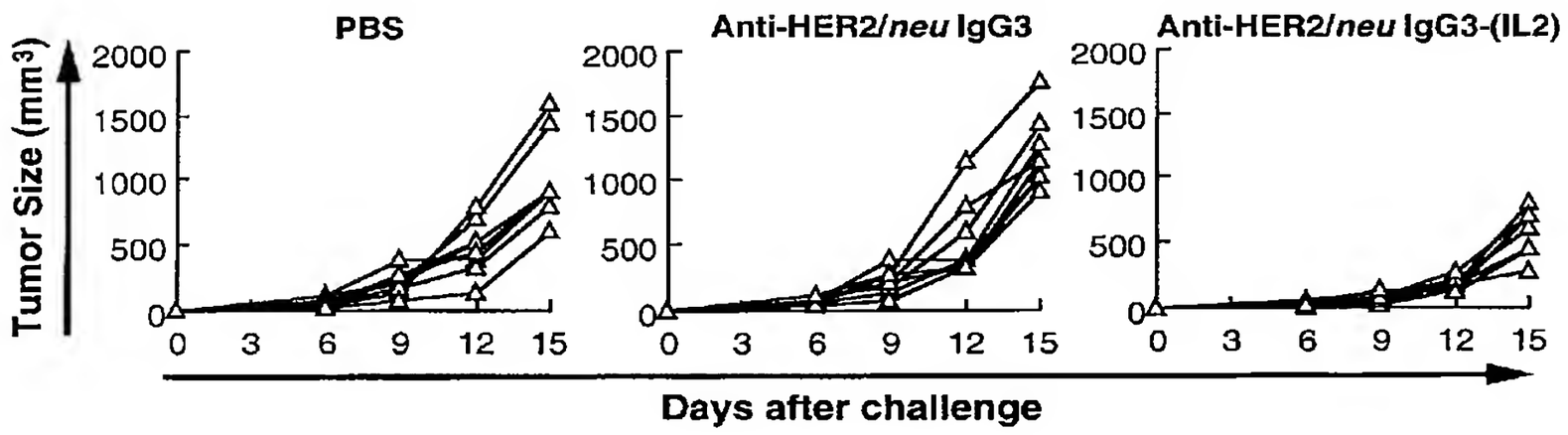

Fig. 1. Anti-Tumor Activity of anti-HER2/*neu* IgG3-(IL-2) and anti-HER2/*neu* IgG3. 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15. The volume was calculated for each mouse of each treatment group, PBS (panel A), anti-HER2/*neu* IgG3 (panel B), and anti-HER2/*neu* IgG3-(IL-2) (panel C).

randomized and groups of 8 received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was monitored and measured with a caliper every three days until day 15 at which time mice were euthanized and serum samples collected. Injection of anti-HER2/*neu* IgG3- (IL-2) results in a significant retardation in the tumor growth in most of the mice as compared with the respective controls of PBS or anti-HER2/*neu* IgG3 (Fig. 1). A two-tailed Student's t-test comparing the tumor volume for each mouse from the group treated with anti-HER2/*neu* IgG3-(IL-2) fusion protein with the mice from the group treated with PBS or anti-HER2/*neu* IgG3 showed that the tumor sizes were statistically different ($p < 0.05$) for all the observed points: days 6, 9, 12, and 15 (Table 1). There was no statistically significant difference in tumor volume between the groups injected with PBS and anti-HER2/*neu* IgG3.

Mice treated with anti-HER2/*neu* IgG3-(IL-2) exhibited a significantly increased ($p < 0.01$) antibody response to anti-HER2/*neu* human IgG3 compared to mice treated with anti-HER2/*neu* IgG3 (Table 2). Mice treated with anti-HER2/*neu* IgG3-(IL-2) showed higher levels of antibodies of all isotypes recognizing human IgG3 when compared to anti-HER2/*neu* IgG3 treated mice (Fig. 2).

4. Discussion

In an attempt to improve the clinical efficacy of anti-HER2/*neu* based therapies we have developed an alternative approach in which human IgG3 containing the variable regions of trastuzumab has been genetically fused to immunostimulatory molecules such as the cytokine IL-12 [30], the costimulatory molecule B7.1 [31], and now IL-2. Targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion proteins recognizing TAAs has been an effective approach to specifically eliminate many tumors [23,26].

A number of factors were considered in the design of our anti-HER2/*neu* IgG3-(IL-2) fusion protein. Human IgG3 was chosen because its extended hinge region should provide spacing and flexibility thereby facilitating simultaneous antigen and receptor binding [32,33] IgG3 is also effective in complement activation [34], and binds FcγRs [34]. IL-2 was used because of its potent immunostimulating properties [17–20,35] and because targeting IL-2 to the site of a tumor with an antibody-(IL-2) fusion protein recognizing TAAs has been an effective approach for specifically eliminating many tumors [23–26]. Antibody-(IL-2) fusion proteins recognizing TAAs have shown superior anti-cancer activity compared with an equivalent amount of free antibody and IL-2 or non-tumor specific antibody cytokine fusion proteins [23,26]. Human IL-2 was used so that the resulting fusion protein was mostly human. Human IL-2 is active in mice making it possible to perform in vivo studies using immune competent mice bearing human HER2/*neu* expressing tumors.

A a single chain Fv specific for peptide epitopes of HER2/*neu* presented by HLA-A*0201 molecules genetically fused to IL-2 [36] (neu-Ab-IL-2) was found to enhance tumor cell eradication by HER2/*neu*-specific $CD8^+$ T cells in an adoptive transfer model in SCID mice. Surprisingly, the combination of non-tumor-specific $CD8^+$ T cells and fusion protein also induced a significant delay of tumor growth, suggesting the potential use of this molecule for redirecting non-tumor-specific T cells to eliminate tumors [36]. However,

Table 1
Mean tumor volumes and statistical significance

Days after the Challenge	Mean Tumor Volumes (mm^3)[a]			Significance[b]	
	PBS	IgG3	IgG3-IL-2	(p) 1	(p) 2
6	67	83	23	0.0114	0.0001
9	221	221	80	0.0070	0.0007
12	479	535	188	0.0013	0.0063
15	1006	1217	571	0.0054	0.0001

[a] 10^6 CT26-HER2/*neu* cells were injected s.c. into the right flank of BALB/c mice. Beginning the next day groups of 8 mice received five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every three days until day 15 and the volume was calculated for each mouse of each treatment group. Mean Tumor Volumes respresents the average tumor volume for each treatment group.
[b] Statistical analysis of the anti-tumor experiments was done using a two-tailed Student's t-test. For all cases results were regarded significant if p values were $\leqslant 0.05$. (p) 1 and (p) 2 represent the p obtained when Mean Tumor Volumes of the group injected with anti-HER2/*neu* IgG3-(IL-2) were compared with PBS and anti-HER2/*neu* IgG3 controls respectively.

Table 2
Murine anti-human IgG3 titers[a]

Treatment	Mouse Number							
	1	2	3	4	5	6	7	8
IgG3	450	450	150	150	450	150	150	150
IgG3-(IL2)	1350	1350	450	1350	450	450	450	450

[a] Groups of 8 mice injected s.c. with 10^6 CT26-HER2/*neu* cells were treated beginning the next day with five daily i.v. injections of 0.25 ml of PBS containing 20 μg of anti-HER2/*neu* IgG3-(IL2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Mice were bled 15 days after the injection of the tumor cells and the sera analyzed by a titration ELISA using plates coated with human IgG3. The presence of antibodies was detected using AP-labeled anti-mouse IgG. Values represent the average of duplicate dilutions of serum required to yield an absorbance of 0.1 (410 nm) after 1 hr of incubation.

in contrast with immunoglobulins such as IgG3, scFvs do not have an Fc. Fc associated funtions such as ADCC (an activity that can be enhanced by the presence of IL-2) may play a role in the anti-tumor activity of recombinant antibodies or antibody-(IL-2) fusion proteins. In fact, ADCC has been proposed as a possible mechanism for the clinical response observed with trastuzumab (Herceptin, Genentech, San Francisco, CA) [15]. In addition, studies from other laboratories have shown that while a mouse-human chimeric anti-Id IgG1-mouse IL2 fusion protein (chS5A8-IL2) was effective in the in vivo eradication of the 38C13 tumor, an anti-Id scFv-IL2 fusion protein (scFvS5A8-IL2) containing the variable regions of the chS5A8-IL2 failed to confer protection. These studies suggested that the Fc effector functions such as ADCC contributed to the anti-tumor activity against 38C13 [24]. It is therefore possible that an anti-HER2/*neu* IgG3-(IL-2) will be superior to anti-HER2/*neu* scFv-(IL-2) in its anti-tumor activity.

We have found that treatment with anti-HER2/*neu* IgG3-(IL-2) causes a significant retardation in the growth of s.c. CT26-HER2/*neu* tumors under conditions in which anti-HER2/*neu* failed to confer protection. However, we did not observe complete tumor eradication in any mice. Several factors could explain the failure to obtain complete tumor remission. The dose, route and schedule of treatment (daily i.v. injection of 20 μg for 5 days) may not be the optimal and/or the tumor model may not be ideal. In addition, we found that treatment with anti-HER2/*neu* IgG3-(IL-2) results in a dramatic increase in the murine anti-human antibody (MAHA) response. This humoral immune response may be sufficient to neutralize multiple injections of the antibody fusion protein.

It is possible that that an anti-human IgG immune response will not pose a problem in humans, and anti-HER2/*neu* IgG3-(IL-2) may be even more effective in patients than in mice. However, the IL-2 in the antibody fusion protein may act as an adjuvant to elicit an im-

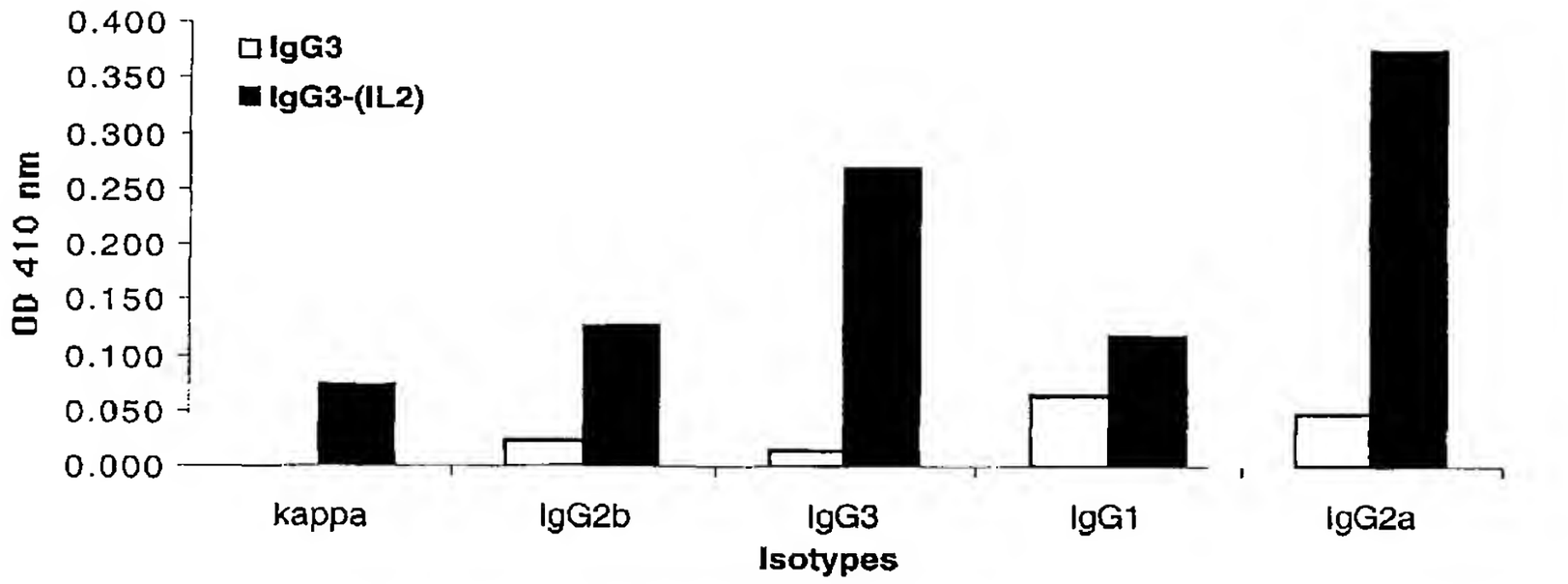

Fig. 2. Isotype profile of antibodies specific for human IgG3. Pooled sera (1/50 dilution) from mice treated with anti-HER2/*neu* IgG3 (clear bar), or anti-HER2/*neu* IgG3-(IL-2) (black bar) were analyzed by ELISA for antibodies of different isotypes recognizing anti-HER2/*neu* IgG3.

mune response against the variable regions of humanized or chimeric antibodies. In addition, even though each component of the antibody-(IL-2) fusion protein may not be antigenic by itself in humans, the novel combination of components may produce neoantigenic determinants that will elicit an immune response. Although in certain cases this enhancement of the immune response may be a serious drawback for the therapeutic use of antibody-(IL-2) fusion proteins, in other cases it may be irrelevant [37] or even an advantage [38,39].

In conclusion, our results suggest that an anti-HER2/*neu* IgG3-(IL-2) fusion protein containing human IL-2 may be an effective therapeutic in patients with tumors overexpressing HER2/*neu*. The combination of an anti-HER2/*neu* antibody with IL-2 yields a protein with the potential to eradicate tumor cells by a number of mechanisms including the down regulation of HER2/*neu* expression, ADCC and the stimulation of a strong anti-tumor immune response through the immunostimulatory activity of IL-2. In addition, the anti-HER2/*neu* IgG3-(IL-2) fusion protein may be effective against tumor cells which express a truncated form of ECD^{HER2} lacking the receptor function rendering them particularly resistant to anti-HER2/*neu* antibody therapy [14]. Because of IL-2's ability to elicit an immune response to associated antigens (as observed with the dramatically increased immune response against human IgG3), it is also possible that association of anti-HER2/*neu* IgG3-(IL-2) with soluble ECD^{HER2} shed by tumor cells will enhance the anti-tumor immune response against ECD^{HER2}. Secretion of ECD^{HER2} has been reported to be a serious drawback for anti-HER2/*neu* therapy in humans [14,15].

Finally we would like to stress that anti-HER2/*neu* IgG3-(IL-2) would not be a replacement for trastuzumab (Herceptin, Genentech, San Francisco, CA), but instead would provide an alternative therapy to be used in combination with the antibody or other anti-cancer approaches. These approaches might include chemotherapy or other anti-HER2/*neu* antibody fusion proteins such as anti-HER2/*neu* with the costimulator B7.1 [31] or the cytokine IL-12 [30]. The availability of more than one antibody fusion protein will allow us to explore potentials synergistic effects that may be obtained from manipulating the immune response.

Acknowledgments

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Induction of Antigen-Specific Cytotoxic T Lymphocytes in Humans by a Malaria DNA Vaccine

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CD8$^+$ cytotoxic T lymphocytes (CTLs) are critical for protection against intracellular pathogens but often have been difficult to induce by subunit vaccines in animals. DNA vaccines elicit protective CD8$^+$ T cell responses. Malaria-naïve volunteers who were vaccinated with plasmid DNA encoding a malaria protein developed antigen-specific, genetically restricted, CD8$^+$ T cell–dependent CTLs. Responses were directed against all 10 peptides tested and were restricted by six human lymphocyte antigen (HLA) class I alleles. This first demonstration in healthy naïve humans of the induction of CD8$^+$ CTLs by DNA vaccines, including CTLs that were restricted by multiple HLA alleles in the same individual, provides a foundation for further human testing of this potentially revolutionary vaccine technology.

During 1990–1994, the administration of "naked" plasmid DNA encoding a specific protein antigen was shown to induce expression of the protein in mouse myocytes (*1*), to elicit antibodies against the protein (*2*), and to manifest protection against influenza (*3*) and malaria (*4*) that was dependent on CD8$^+$ T cell responses against the expressed protein. Hundreds of publications have now reported the efficacy of DNA vaccines in small and large animal models of infectious diseases, cancer, and autoimmune diseases (*5*).

DNA vaccines elicit antibodies and CD4$^+$ T cell responses in animals, but their major advantage at the immunological level has been their capacity to induce antigen-specific CD8$^+$ T cell responses, including CTLs, which is a major mechanism of protection against intracellular pathogens. Important to our method of developing a malaria vaccine is the induction of CD8$^+$ T cell responses against *Plasmodium falciparum*–infected hepatocytes (*6*). The lysis of cells in a standard chromium release assay was used as a surrogate for antihepatocyte responses, because it has been established that CD8$^+$ CTLs, which recognize peptide-pulsed target cells, also recognize and eliminate parasite-infected hepatocytes (*6*). On the basis of our work with rodents (*4*, *7*) and our work and that of others with rhesus monkeys (*8*, *9*), we have developed a plan for manufacturing and testing the efficacy of a multigene *P. falciparum* liver-stage DNA vaccine in humans (*10*). This has been contingent on establishing that DNA vaccination of humans is safe and induces antigen-specific, genetically restricted, CD8$^+$ T cell–dependent CTLs. Recently, the presence of CTL responses in human immunodeficiency virus (HIV)–infected individuals after vaccination with plasmid DNA encoding the *nef*, *rev*, or *tat* genes or the *env* and *rev* genes of HIV was reported (*11*). Interpreting these results is difficult because of the concurrent HIV infection, which has been demonstrated to prime individuals for a CTL response that is independent of immunization.

Accordingly, 20 healthy, malaria-naïve adults were recruited and randomized into four dosage groups of five individuals. Three injections of 20, 100, 500, or 2500 μg of plasmid DNA encoding the *P. falciparum* circumsporozoite protein (PfCSP) (*12*) were administered at 4-week intervals in alternate deltoids (*13*). The details of recruitment, safety, and tolerability were reported elsewhere (*14*). To assess CTL responses, we collected peripheral blood mononuclear cells (PBMCs) from each volunteer before vaccination, 2 weeks after the second immunization, and 2 and 6 weeks after the third immunization. These cells were either assayed while fresh for recall antigen-specific CTL responses (*15*) or were frozen (*16*) for subsequent study. In parallel, CTL assays were carried out with PBMCs from nonimmunized control volunteers. Cytolytic activity was assessed after both primary and secondary in vitro restimulation against HLA-matched and HLA-mismatched PfCSP-specific and control targets. The percent lysis and the percent specific lysis were determined as described (*15*). The most sensitive and specific method (*17*) for demonstrating the presence of CTLs was with effector cells that were expanded in vitro by exposure to cells infected with canary pox (ALVAC) expressing the PfCSP (*18*) and with target cells that were sensitized with PfCSP-derived synthetic peptides (*19*). There was no apparent difference between the primary and secondary assays (*20*) or between the fresh and frozen specimens (*21*).

For logistical reasons, fresh PBMCs were studied only before vaccination and after the second immunization in the 20- and 100-μg-dosage groups but were studied before vaccination and after all immunizations in the 500- and 2500-μg-dosage groups, with the exception of one individual (*13*). For 14 individuals, adequate amounts of frozen PBMCs were available for further analysis. A typical pattern of CTL responses is presented in Fig. 1A. These responses were peptide-specific and genetically restricted because there was little or no recognition of autologous targets that were incubated with the control peptide or of HLA class I–mismatched targets that were incubated with the specific peptide. This activity was shown to be CD8$^+$ T cell–dependent by restimulating

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the effector cells and repeating the assay after the depletion of T cell subsets (Fig. 1B) (*22*). The simultaneous assessment of coded frozen PBMCs that were collected before and after vaccination (Fig. 1C) confirmed that these CTLs were induced after vaccination with a plasmid DNA and after subsequent translation of the encoded PfCSP.

The CTL responses with fresh PBMCs from 9 of 20 volunteers and with frozen PBMCs from 6 of 14 volunteers met our criteria for positivity. Eleven of 20 volunteers were shown to have antigen-specific, genetically restricted CTL activity. The effect of T cell subset depletion was studied in fresh cells that were acquired from volunteers in the 500- and 2500-μg-dosage groups after the third immunization. In the five responders tested, $CD8^+$ T cell depletion eliminated the CTL activity (Fig. 1B), and $CD8^+$ T cell dependence was demonstrated for all 10 peptides, except peptide $B35_{353}$. Volunteer 33 was the only volunteer who expressed HLA-B35. Accordingly, peptide $B35_{353}$ was tested only once [at 2 weeks after immunization (*13*)]—at which time peptide-specific, genetically restricted CTLs were detected, but $CD8^+$ dependence was not assessed. The presence of $CD4^+$ CTLs could not be completely excluded, because (in some cases) there was a reduction in cytolytic activity upon the depletion of $CD4^+$ T cells. However, this reduction was minor in relation to the effect of $CD8^+$ T cell depletion (Fig. 1B).

In nine volunteers, CTLs could not be detected in the three assays that were conducted after immunization. In three of these volunteers, the lack of response could not be attributed to a failure to respond to the vaccine, because these individuals did not express any of the HLA alleles restricting the peptides under study (volunteers 8 and 19, 20 μg; volunteer 20, 500 μg). Four of the other six nonresponders were in the two lower dosage groups.

The CTL responses of all volunteers to all peptides after immunization are summarized in Table 1. The representative data for each responder to each peptide are presented in Fig. 2. The responses after immunization (80 of 341

Table 1. Summary of overall CTL responses as assessed by vaccinia-stimulated effectors and peptide-sensitized targets with fresh and frozen PBMCs. Numbers separated by slashes without parentheses indicate the number of positive peptides out of the number of tested peptides; numbers separated by slashes in parentheses indicate the number of positive assays out of the number of assays. A positive assay result is defined as a percent specific lysis after vaccination that is >10% for at least two E:T ratios in the same assay, with a percent specific lysis before vaccination of <10%. In volunteers 7, 5, 13, and 37, CTLs against some peptides were detected before vaccination; these peptides were excluded from analysis for the respective individuals. nt, not tested.

Group data	2 weeks after second immunization	2 weeks after third immunization	6 weeks after third immunization	Total	Range of percent specific lysis
		Group 1 (20 μg)			
Volunteer code					
7	2/4 (2/8)	4/7 (4/7)	0/7 (0/7)	5/7 (6/22)	10.0–33.7
8	0/1 (0/2)	0/1 (0/1)	0/1 (0/1)	0/1 (0/4)	
12	0/2 (0/4)	nt	1/2 (1/2)	1/2 (1/6)	12.4–22.6
13	0/5 (0/8)	0/6 (0/6)	0/6 (0/6)	0/6 (0/20)	
19	0/1 (0/2)	0/1 (0/1)	0/1 (0/1)	0/1 (0/2)	
Overall	2/13 (2/24)	4/15 (4/15)	1/17 (1/17)	6/17 (7/54)	10–22.6
Percentage of positive peptides (percentage of positive assays)	15.4% (8.3%)	26.7% (26.7%)	5.9% (5.9%)	35.3% (13.0%)	
		Group 2 (100 μg)			
Volunteer code					
5	0/4 (0/4)	3/6 (3/6)	1/6 (1/6)	4/6 (4/16)	10.0–16.1
6	2/4 (2/8)	nt	nt	2/4 (2/8)	10.0–22.9
18	0/2 (0/4)	0/2 (0/2)	0/2 (0/2)	0/2 (0/8)	
22	0/4 (0/4)	0/5 (0/5)	0/5 (0/5)	0/5 (0/14)	
26	0/4 (0/8)	0/6 (0/6)	0/1 (0/1)	0/6 (0/15)	
Overall	2/18 (2/28)	3/19 (3/19)	1/14 (1/14)	6/23 (6/61)	10.0–22.9
Percentage of positive peptides (percentage of positive assays)	11.1% (7.1%)	15.8% (15.8%)	7.1% (7.1%)	26.1% (9.8%)	
		Group 3 (500 μg)			
Volunteer code					
20	0/1 (0/2)	0/1 (0/2)	0/1 (0/2)	0/1 (0/6)	
23	0/4 (0/8)	0/5 (0/8)	4/5 (4/13)	4/5 (4/29)	10.8–26.7
27	0/3 (0/6)	2/4 (2/8)	4/4 (4/11)	4/4 (6/25)	10.7–51.9
36	0/4 (0/8)	6/7 (12/14)	6/7 (6/17)	7/7 (18/39)	10.9–55.4
40	0/4 (0/8)	0/4 (0/8)	0/5 (0/10)	0/5 (0/26)	
Overall	0/16 (0/32)	8/21 (14/40)	14/22 (14/53)	15/22 (28/125)	10.7–55.4
Percentage of positive peptides (percentage of positive assays)	0% (0%)	38.1% (35.0%)	63.6% (26.4%)	68.2% (22.4%)	
		Group 4 (2500 μg)			
Volunteer code					
21	0/2 (0/4)	0/2 (0/4)	0/2 (0/4)	0/2 (0/12)	
29	1/1 (1/2)	0/1 (0/2)	0/1 (0/2)	1/1 (1/6)	16.6–28.5
33	4/4 (4/8)	nt	nt	4/4 (4/8)	10.1–31.1
37	1/4 (1/8)	1/6 (3/16)	3/7 (6/17)	3/7 (9/41)	10.0–67.73
39	4/4 (6/8)	5/5 (6/10)	5/5 (7/14)	5/5 (19/32)	10.5–37.9
Overall	10/15 (12/30)	6/14 (9/32)	8/15 (13/37)	13/17 (33/99)	10.0–67.73
Percentage of positive peptides (percentage of positive assays)	76.9% (40%)	42.9% (28.1%)	53.3% (35.1%)	76.5% (33.3%)	

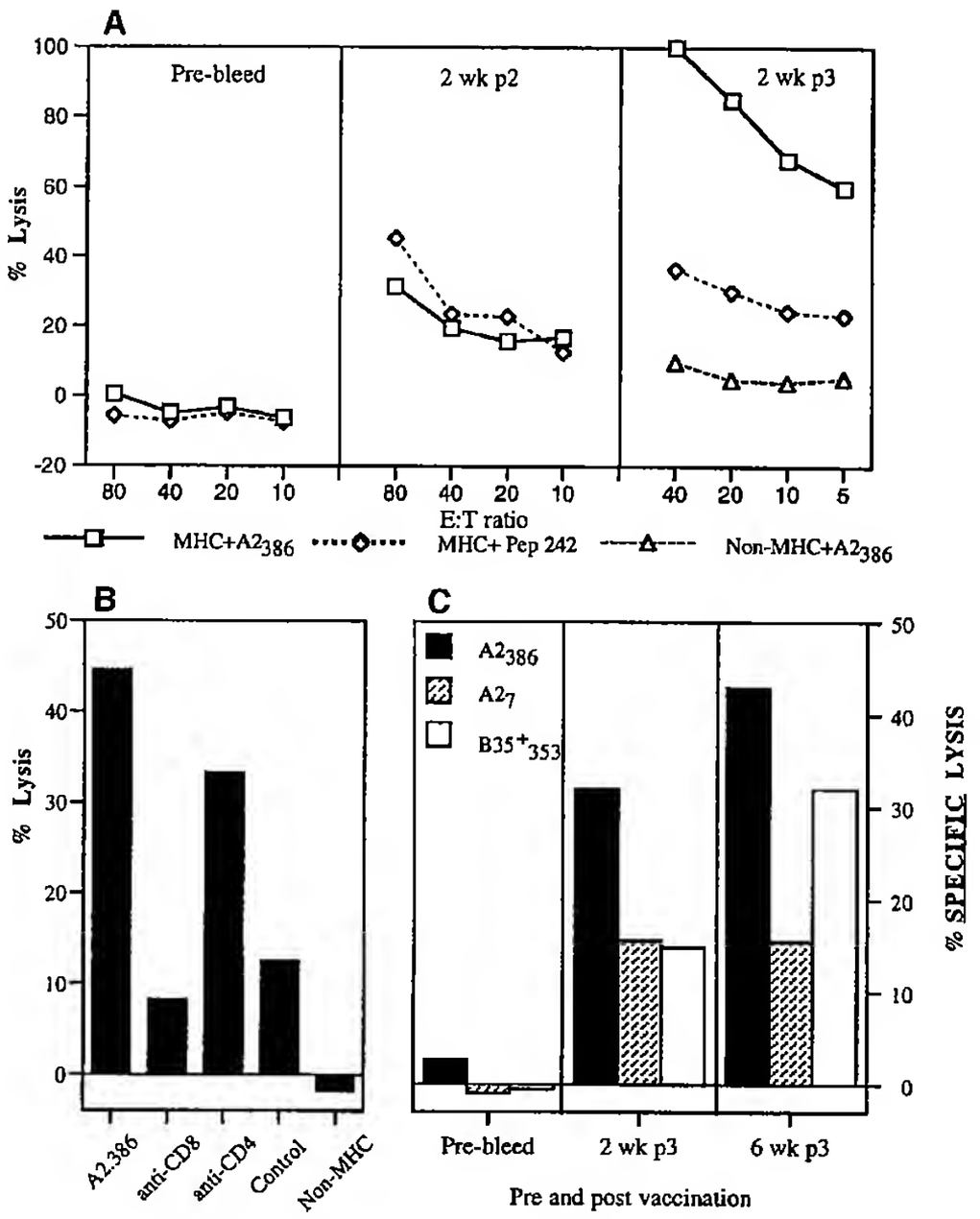

Fig. 1. (**A**) Peptide-specific recall CTL response to vaccination with plasmid DNA. Fresh PBMCs from volunteer 37 (2500-μg-dosage group) [which were collected before vaccination (pre-bleed), 2 weeks after the second immunization (2 wk p2), and 2 weeks after the third immunization (2 wk p3)] were stimulated in vitro with ALVAC expressing the PfCSP for 7 days and then were assayed against HLA class I–matched or HLA class I–mismatched PfCSP peptide–pulsed or control peptide 242–pulsed targets in a conventional chromium release assay (*15*). MHC, major histocompatibility complex. (**B**) DNA-induced CTL responses are $CD8^+$ T cell–dependent. ALVAC PfCSP–stimulated effector cell populations from volunteer 37 were depleted in vitro of $CD8^+$ or $CD4^+$ T cells (*22*) immediately before the chromium release assay. Results of a 13-day assay at an E:T ratio of 20:1 are presented. (**C**) DNA-induced CTL responses with frozen PBMCs (*16*). Coded frozen PBMCs from volunteer 37 were assayed for peptide-specific CTLs. The % specific lysis in a 7-day assay at an E:T ratio of 20:1 is presented.

assays, 23.4%) were significantly greater than those before immunization (6 of 139 assays, 4.3%) ($P = 0.0000007$, chi-squared test). Two of the 82 assays (2.4%) that were conducted with PBMCs from control (nonimmunized) volunteers were positive, which is significantly less than those assays that were conducted with PBMCs after immunization ($P = 0.000015$).

An apparent positive response was noted with one peptide for volunteers 5 (1 of 12 assays), 7 (1 of 10 assays), and 13 (1 of 8 assays) and with three peptides for volunteer 37 (3 of 11 assays) in secondary but not primary assays of fresh PBMCs that were collected before immunization. However, CTL responses after vaccination were significantly enhanced in relation to the levels before vaccination. Furthermore, no activity was detected when the assay was repeated with frozen PBMCs (*21*). Nevertheless, in accordance with our conservative definition of positivity, all peptides with ≥10% specific lysis before vaccination were excluded from subsequent analysis for the respective individual.

Peptide-specific, genetically restricted, and $CD8^+$ T cell–dependent CTL responses were induced by as little as two 20-μg doses of DNA (Table 1). The induction of CTLs after a single immunization was not tested. CTL responses were detected in two of five volunteers immunized with 20 μg of DNA or 100 μg of DNA, in three of five volunteers immunized with 500 μg of DNA, and in four of five volunteers immunized with 2500 μg of DNA. Data indicate that immunization with either 500 or 2500

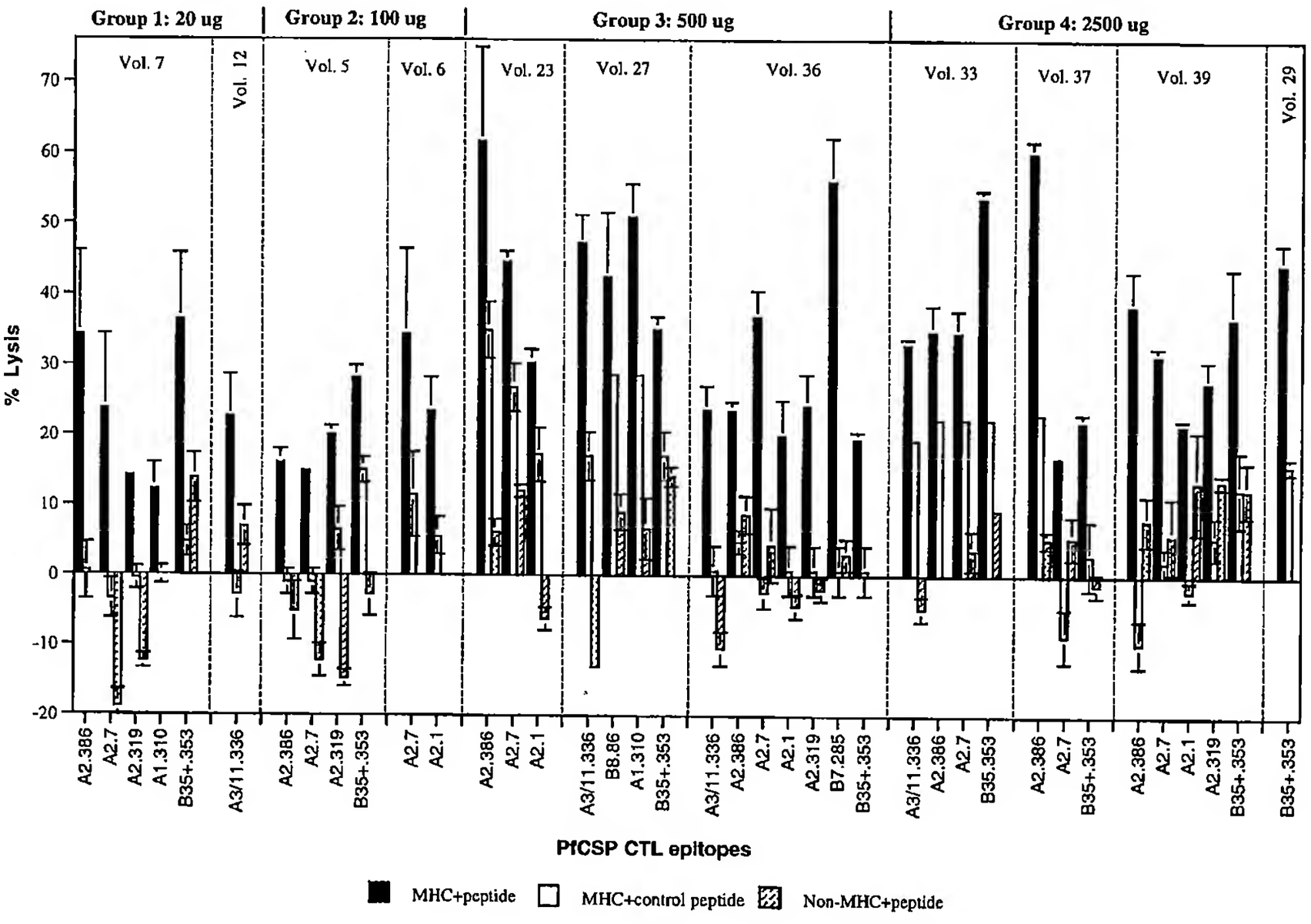

Fig. 2. Representative data of positive (difference between the percent lysis of target cells pulsed with experimental or control peptides ≥10%) CTL responses for each volunteer for each peptide. Fresh or frozen PBMCs, taken at the same or different time points, were assayed for peptide-specific, genetically restricted CTLs as described in the caption of Fig. 1A. Shown is the percent lysis (mean ± SEM) for each peptide with its simultaneously assessed controls at a single E:T ratio. Error bars indicate SEM.

μg of DNA induced a significantly better CTL response in comparison with either 20 or 100 μg of DNA ($P \leq 0.0003$). There was no significant difference between 500- and 2500-μg dosages overall or after the third immunization ($P \geq 0.53$), but a significantly higher frequency of response was induced with 2500 μg of DNA in comparison to 500 μg of DNA after the second immunization ($P = 0.000001$). There was no significant difference between 20- and 100-μg dosages at any time ($P \geq 0.43$).

With regard to the immunization schedule, overall, the rate of positive assays 2 weeks after the third immunization (31 of 106) was significantly greater than the rate after the second immunization (19 of 114) ($P = 0.026$); there was no significant difference between the 2- and 6-week time points after the third immunization (31 of 106 versus 30 of 121) ($P = 0.45$) (Table 1).

The frequency and magnitude of the CTL responses to specific peptides are summarized in Table 2. Overall, 5 of the 11 responders recognized 100% of the peptides studied, 3 responders recognized 60 to 70% of the peptides, and 3 others recognized 43 to 50% of the peptides.

The DNA-induced CTLs were genetically restricted by multiple HLA alleles (Table 2). Representative data are presented in Fig. 3. There was no apparent hierarchy in terms of allele-specific recognition. The magnitude of the induced CTL responses to defined epitopes varied between volunteers. Overall, the best response was detected for the HLA-A2 restricted epitope, $A2_{386}$ (Table 2). This response was not substantially different than the responses that were noted for the peptides $A1_{310}$, $A2_7$, $A3/11_{336}$, $B7_{285}$, $B35_{353}$, and $B35+_{353}$. Induction by subunit vaccines of $CD8^+$ T cell–dependent immune responses of multiple HLA restrictions in the same individual has, to our knowledge, not previously been reported for any infectious agent in humans. This has been a major obstacle to vaccine development and will be critical to the success of a malaria vaccine, because T cell responses to individual epitopes are genetically restricted and there is a substantial allelic variation of CTL epitopes among *P. falciparum* isolates in nature (*23*). Indeed, it has been demonstrated that the irradiated *P. falciparum* sporozoite vaccine, which confers potent protective immunity in humans (*6*), induces CTL responses that are restricted by multiple HLA alleles in genetically diverse individuals (*24*).

In mice, immunization with a *P. yoelii* circumsporozoite protein DNA vaccine elicits a substantially greater CTL response than does immunization with irradiated sporozoites (*4*). We did not simultaneously compare CTL responses in our vaccine recipients with CTL responses in individuals who were immunized with irradiated sporozoites or naturally exposed to malaria. However, as in the rodent model (*4*), the magnitude of the CTL response that was seen in some of the volunteers (Fig. 1) was considerably higher than the response that is generally seen in humans exposed to irradiated sporozoites or to natural infection (*24*–*27*).

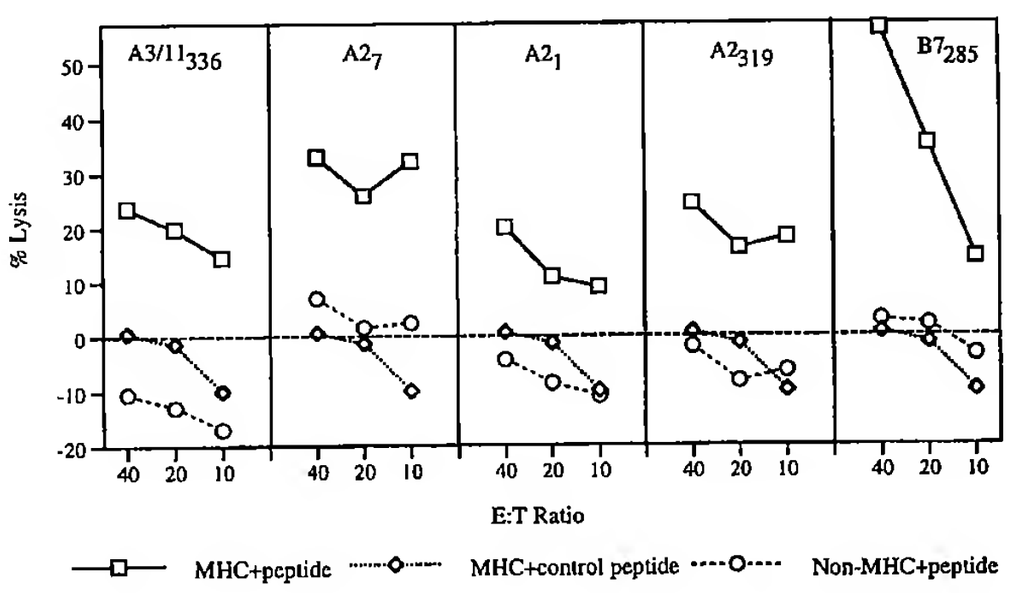

Fig. 3. DNA-induced CTL responses are restricted by multiple HLA alleles. Fresh PBMCs from volunteer 36 (500-μg-dosage group), who expressed the alleles HLA-A2, A3, and B7, were assayed for antigen-specific, genetically restricted CTLs (*15*). The assay was repeated with coded frozen PBMCs (*16*) that were collected before and after vaccination; the results confirmed that the peptide-specific (the same five peptides), genetically restricted CTLs were induced by vaccination with plasmid DNA (*21*).

Table 2. HLA restriction and magnitude and frequency of CTL responses for each of the 10 peptides studied.

Peptide	HLA restriction	Percent specific lysis range	Number of positive assays/total assays (%)	Number of responders/ number tested
$A1_{310}$	A1	12.3–18.0	3/16 (18.8)	2/4
$A2_{386}$	A2 supertype	10.2–67.7	15/42 (35.7)	7/12
$A2_7$	A2 supertype	10.0–36.9	15/46 (32.6)	7/12
$A2_1$	A2.1	10.8–23.2	7/32 (21.9)	4/11
$A2_{319}$	A2.1	10.5–24.6	11/39 (28.2)	5/11
$A3/11_{336}$	A3/11 supertype	10.0–51.9	8/27 (29.6)	4/7
$B7_{285}$	B7	10.2–55.4	3/12 (25.0)	1/3
$B8_{86}$	B8	11.7–14.0	2/9 (22.2)	1/2
$B35_{353}$	B35	23.1–31.4	1/2 (50.0)	1/1
$B35_{+353}$	B35 plus	10.0–37.9	9/61 (14.8)	7/18

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12. The full-length PfCSP gene [1194 base pairs (bp)] from the *P. falciparum* clone 3D7 [J. R. Campbell, *Nucleic Acids Res.* **17**, 5854 (1989)] was cloned into the eukaryotic expression vector VR1020 [C. J. Luke, K. Carner, X. Liang, A. G. Barbour, *J. Infect. Dis.* **175**, 91 (1997)] as an in-frame fusion with the human tissue plasminogen activator leader peptide to create Vical Clinical plasmid VCL-2510. Clinical supplies and a qualification of this construct were produced under good manufacturing practices (S. E. Parker *et al.*, in preparation). Four vaccine formulations were stored as 1.0-ml doses at −20°C and then thawed at room temperature for 30 min before injection. In vitro expression and in vivo immunogenicity of VCL-2510 in rodents and nonhuman primates have been reported elsewhere (*8*) [R. C. Hedstrom *et al.*, *Int. J. Mol. Med.* **2**, 29 (1998).
13. The study was a dose-escalating phase I safety and immunogenicity trial in healthy adult volunteers with informed consent. A total of 28 healthy, malaria-naïve volunteers were selected for the study on the basis of negative serologic studies for PfCSP, HIV, hepatitis B virus, hepatitis C virus, and smallpox. The volunteers were between 20 and 29 years old, and 61% were male. Complete class I and class II HLA typing profiles were obtained (*21*). Eight volunteers did not receive the PfCSP DNA vaccine and served as assay controls. Volunteer 33 (2500-μg-dosage group) was withdrawn from study after the second immunization because of an unplanned pregnancy.
14. T. P. Le *et al.*, in preparation.
15. PBMCs were cultured in RPMI 1640 supplemented with 10 mM Hepes, 2 mM L-glutamine, penicillin (50 U/ml), streptomycin (50 μg/ml) (Life Technologies, Grand Island, NY), and 10% heat-inactivated type AB human serum (ICN Biomedical, Costa Mesa, CA). To generate effector cells, we infected 20% of the total PBMCs with ALVAC expressing the PfCSP (vCP182) (*18*) at 5 plaque-forming units per cell for 90 min at 37°C; these PBMCs were washed twice, combined with the remaining PBMCs, and cultured at 3×10^6 cells per 2 ml in 24-well plates for 7 days. Recombinant human interleukin-2 (rIL-2) (Cetus, Emeryville, CA) was added after 48 hours (20 U/ml). For secondary stimulation (13-day assay), rIL-7 (Peprotech, Rocky Hill, NJ) was added at the

initiation of the culture at 100 U/ml. On day 8, 6×10^6 to 10×10^6 responder cells were incubated with 1×10^7 autologous phytohemagglutinin (PHA) blasts infected with vCP182 and were cultured at 1×10^6 cells/ml in a 25-cm^2 flask in the presence of rIL-2 (20 U/ml) and rIL-7 (100 U/ml) for an additional 5 days. PHA blasts were generated by stimulating PBMCs with 0.2% (v/v) PHA (Sigma). Primary CTL assays were performed on day 7. Secondary CTL assays were performed on day 13 after restimulation on day 8. Target cells were autologous or HLA-mismatched PHA blasts that were sensitized overnight with synthetic peptides (10 μg/ml) representing previously identified CTL epitopes on the PfCSP (*19*). Targets were labeled with 100 μCi ^{51}Cr [sodium chromate solution (Dupont New England Nuclear, Boston, MA)] for 90 min at 37°C and washed three times before use. The CTL activity was assessed by a conventional 6-hour chromium release assay, in the presence of a peptide (10 μg/ml). The percent lysis was defined as (experimental release − medium control release)/(maximum release − medium control release) × 100. The percent specific lysis was determined by subtracting the percent lysis of the targets that were sensitized with control peptide 242 from the percent lysis of the targets that were incubated with the experimental peptide. The results were expressed as the mean of triplicate determinations. The CTL responses were considered to be positive only if the percent specific lysis after immunization was ≥10% for at least two effector:target (E:T) ratios in the same assay and if the percent specific lysis before immunization was <10%. Spontaneous release values were always <20%.

16. PBMCs were resuspended at a concentration of 10×10^6 cells/ml in 20% fetal calf serum (Sigma) in RPMI 1640, and an equal volume of ice-cold 15% dimethyl sulfoxide in RPMI 1640 was added dropwise, with shaking. All procedures were performed on ice. The cells were transferred to cryotubes at a final concentration of 5×10^6 cells/ml in each vial, and the tubes were placed in a plastic foam container at −80°C overnight before being transferred to liquid nitrogen.
17. The CTL assays were conducted with four E:T combinations: (i) ALVAC PfCSP effectors and Western Reserve (WR) vaccinia PfCSP targets, (ii) ALVAC PfCSP effectors and experimental or control peptide–pulsed targets, (iii) experimental peptide–induced effectors and WR vaccinia PfCSP targets, and (iv) experimental peptide–induced effectors and experimental or control peptide–pulsed targets. All assays with WR vaccinia PfCSP-infected targets were excluded from the analysis because a simultaneous assay of PBMCs from control-naïve volunteers demonstrated an unacceptably high level of positivity (*21*). Assays that were conducted with PfCSP peptide-stimulated effectors against peptide-sensitized targets were not positive.
18. Recombinant pox viruses were produced in collaboration with Virogenetics (Troy, NY) [J. A. Tine *et al.*, *Infect. Immun.* **64**, 3833 (1996); D. E. Lanar *et al.*, *ibid.*, p. 1666]. The ALVAC virus expressing PfCSP (vCP182) was used for the stimulation of CTL effectors. Recombinant vaccinia viruses (WR) encoding PfCSP (vP1255) or PfLSA-1 (vP1253) (control) were used for the infection of target cells.
19. The following peptide sequences and residue numbers are based on the complete PfCSP 3D7 amino acid sequence (residues 1 through 397), and variant residues are indicated in bold and underlined: (i) $A1_{310}$, HLA-A1 restricted, residues 310 through 319, sequence EPSDKHI**K**E**Y** (*28*, *29*); (ii) $A2_{386}$, HLA-A2 supertype, residues 386 through 394, GLIMVLSFL (*24*); (iii) $A2_7$, HLA-A2 supertype, residues 7 to 16, ILSVSSFLFV (*24*); (iv) $A2_1$, HLA-A2.1, residues 1 through 10, MMRKLAILSV (*30*); (v) $A2_{319}$, HLA-A2.1, residues 319 through 327, YL**N**KI**Q**NSL (*30*); (vi) $A3/11_{336}$, HLA-A3/11 supertype, residues 336 through 345, VTCGNGIQVR (*24*); (vii) $B7_{285}$, HLA-B7, residues 285 through 293, MPN**D**PNRNV (*25*); (viii) $B8_{86}$, HLA-B8, residues 86 through 94, LRKPKHKKL (*25*); (ix) $B35_{353}$, HLA-B35, residues 353 through 360, K**P**-KDELDY (*26*); (x) $B35+_{353}$, HLA-B35 plus (contains a HLA-B35 restricted epitope but also contains one or two additional epitopes of undefined genetic restriction), residues 353 through 375, K**P**KDELDYANDIEK-KICKKMEKCS (*27*); (xi) Pep242, random sequence, RALMSMVLIK. PfCSP-derived synthetic peptides were synthesized by Pasteur-Merieux Connaught Laboratories and were purified through high-performance liquid chromatography. Control peptide 242 was generated by a random scrambling of the HLA-A2 binding peptide, $A2_1$. Lyophilized peptides were reconstituted at 20 mg/ml with 100% dimethyl sulfoxide (Sigma) and stored at −80°C until use. The peptide was diluted to 2 mg/ml with RPMI 1640 without serum before use.
20. A comparison of the primary assay (one in vitro restimulation) versus the secondary assay (two in vitro restimulations) gave the following results: The number of positive individuals out of the total number of tested individuals was 10 of 20 versus 6 of 20; the number of positive assays out of the total number of assays was 52 of 218 (22.8%) versus 28 of 123 (23.9%); the range of the percent specific lysis was 10.2 to 67.7% versus 10.1 to 37.91%. Volunteer 33, who was not positive in the primary assay, was only studied at one time point (*13*).
21. R. Wang *et al.*, data not shown.
22. The ALVAC PfCSP–stimulated effector cell populations were depleted in vitro of $CD8^+$ or $CD4^+$ T cells immediately before the assay with anti-$CD4^+$– or anti-$CD8^+$–coated Dynabeads M-450, according to the manufacturer's instructions (Dynal, Great Neck, NY). Flow cytometric analysis confirmed that the cell subset depletion was >95% in all cases (*21*).
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29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Differentiation of Monocytes into Dendritic Cells in a Model of Transendothelial Trafficking

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Essential to the dendritic cell system of antigen-presenting cells are the veiled dendritic cells that traverse afferent lymph to enter lymph nodes, where they initiate immune responses. The origin of veiled cells, which were discovered 20 years ago, is unclear. Monocytes cultured with endothelium differentiated into dendritic cells within 2 days, particularly after phagocytosing particles in subendothelial collagen. These nascent dendritic cells migrated across the endothelium in the ablumenal-to-lumenal direction, as would occur during entry into lymphatics. Monocytes that remained in the subendothelial matrix became macrophages. Therefore, monocytes have two potential fates associated with distinct patterns of migration.

One of the important features of dendritic cells (DCs) is their capacity to migrate from peripheral tissues to lymphoid organs and initiate immunity. DCs gain access to the spleen from the bloodstream and enter lymph nodes by migration through afferent lymphatic vessels (*1*). Bearing soluble proteins (*2*) and particulates (*3*) that they acquire before entry into lymph nodes, DCs localize to the T cell areas where they are ideally positioned to select and activate clones of antigen-reactive T lymphocytes. When afferent lymphatic conduits are severed, immunity to peripherally administered antigen does not develop (*4*). Lymph DCs may also induce peripheral tolerance to self-antigens acquired during the

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EXHIBIT

AG

Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients

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Summary

Background DNA vaccination is known to generate immune responses against HIV-1 in animal models. We aimed to assess the efficacy of DNA vaccination in induction of immune responses in HIV-1-infected human beings.

Methods Nine symptom-free HIV-1-infected patients were immunised with DNA constructs encoding the *nef*, *rev*, or *tat* regulatory genes of HIV-1. The patients were selected for having no or low antibody reactivities to these antigens. HIV-1-specific cytotoxic T-lymphocytes (CTLs), precursor frequencies, and antigen-specific proliferative responses were measured before, during, and after three immunisations over 6 months.

Findings Cellular immune reactivities against the HIV-1 regulatory proteins were absent or low before DNA immunisation. DNA vaccination induced detectable memory cells in all patients and specific cytotoxicity in eight patients. CTLs were MHC-class-I restricted and mainly of CD8+ origin. In three patients the cellular activity was transient, decreasing after an initial response.

Interpretation DNA immunisation with HIV-1 genes can induce specific cellular responses in human beings with no apparent side-effects. It is theoretically possible that HIV-1-specific cytotoxic responses to regulatory proteins could lead to infected cells being eliminated before they have released new viral particles. However, it is possible that the patients we selected responded less than would non-selected or non-infected individuals. The small number of patients presented here does not allow generalisation of our findings.

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Introduction

Several studies have highlighted the importance of the response of cytotoxic T lymphocytes (CTLs) to contain the early dissemination of HIV-1 in infected individuals. During the symptom-free phase of infection, HIV-1-specific CTL responses, directed at multiple HIV-1 epitopes, can be detected in peripheral blood as well as by in-vitro stimulation in most individuals.[1–9] Later on, a decrease in this activity coincides with the onset of the symptomatic stage of the infection, suggesting that an increased concentration of cytotoxic cells might help to slow or halt progression.[10]

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An attractive approach to enhancing virus-directed cytotoxicity is provided by a novel class of DNA vaccines that are based on plasmid-borne genes. While most studies have tested immunisation in animals,[11–14] our aim was to investigate the immunogenicity of DNA constructs of the regulatory HIV-1 genes *nef*, *rev*, and *tat* in human beings. The induction of immune responses to HIV-1 by immunisation with these regulatory genes in animals has been described in detail elsewhere.[15–18] We attempted induction of HIV-1-specific CTL responses to these antigens in symptom-free patients infected with HIV-1. The three HIV-1 regulatory genes are expressed early in the life-cycle of the virus, so it is theoretically possible that induction of immune responses against these genes would eliminate infected cells, which express these proteins, before viral particles are released.

Materials and methods

Study design

Nine symptom-free patients infected with HIV-1 were enrolled in the study (table 1). Three patients were immunised with HIV-1 *nef* complementary (c) DNA, three with HIV-1 *rev* cDNA, and three with HIV-1 *tat* cDNA, at days 0, 60, and 180. DNA constructs (100 µg cDNA in distilled water) were administered, without adjuvant, by intramuscular injection into the right deltoid muscle. The plasmid vaccines contained either HIV-1 *nef*, *rev*, or *tat* gene, under the control of the human cytomegalovirus immediate—early promoter.[16] Antibody titres were assessed with peptides and proteins that represent *nef*, *rev*, or *tat* genes. Patients were selected for having no antibody reactivities or low antibody rectivities to Nef (three patients), Rev (three patients), or Tat (three patients). One patient was already receiving antiretroviral treatment and one started during the study. For each group of three patients, two symptom-free patients infected with HIV-1, and two laboratory workers who were seronegative for HIV-1 and who had not received HIV-1-DNA immunisation, were recruited as controls for the measurement of responses to CTLs.

The DNA vaccine study, including plasmid preparations, was approved by the Swedish Medical Products Agency and the ethical committee of the South Hospital, Stockholm. Informed consent was given by all patients.

Measurement of cytotoxicity

B-cell lines were generated from each patient.[6,8] They were used both as target cells for testing cytotoxic effects and as antigen-presenting cells during stimulation of the patient's lymphocyte cell population. Antigen-presenting stimulator cells were obtained by incubation of autologous B-lymphoblastoid cell lines (B-LCLs) with: recombinant vaccinia vectors expressing either the *nef* (modified vaccinia Ankara [MVA]-nef), the *rev* (vaccinia strain TG [VVTG]-rev), or the *tat* (VVTG-tat) gene from the LAI strain of HIV-1 at a multiplicity of infection of 2–5; or HIV-1 pseudotyped with amphotropic murine leukaemia virus (MuLV) 4070 (HIV-1/MuLV).[19] The HIV-1/MuLV pseudotype infection transfers the complete HIV-1 LAI genome to the infected cells because the amphotropic leukaemia envelope associates with an ubiquitous sodium-dependent phosphate ion channel receptor.[19,20] Theoretically, infection by the pseudotype HIV-1/MuLV virus should result in several HIV-1 viral peptides being displayed on the target cells. This virus was chosen because it was thought to best represent in-vivo conditions.

Patient effector lymphocytes were stimulated by autologous

Patient	HLA class I	DNA immunogen	CD4* (10⁶ cells/L)		CD8* (10⁶ cells/L)		Viral load (RNA copies/mL plasma)	
			Before (day 0)	After (day 194)	Before (day 0)	After (day 194)	Before (day 0)	After (day 194)
39	A2†	*nef* cDNA	430	421	440	449	1200	7000
7‡	A9,(24),33,B14,(65),35,Bw4,6	*nef* cDNA	430	396	1000	1033	6200	6200
3§	A2,B7,27	*nef* cDNA	413	419	778	914	9300	75500
25	A3,30,B7,13,Bw4,6	*rev* cDNA	1150	740	778	570	<500	<500
34	A11,19,(29),B17,(58),35	*rev* cDNA	380	372	660	664	9600	3200
37‖	A3,19,(32),B12,(44),35,Cw4,5,Bw4,6	*rev* cDNA	415	400	1523	1830	55500	59600
6	A2,B15,(62),27	*tat* cDNA	410	590	850	1060	2500	4900
29¶	A3,10,B7,27	*tat* cDNA	275	417	559	821	69000	<500
12	A2†	*tat* cDNA	405	530	937	1310	51100	40300

*CD4 and CD8 counts as well as viral load are shown before (day 0) and 14 days after the third immunisation.
†HLA class I typing could only be done for A2 alleles due to the fragility of patient cells.
‡Patient 7 was on antiretroviral therapy (zidovudine+didanosine) at entry into the present study.
§Patient 3 started triple antiretroviral combination therapy 10 months after study entry.
‖Patient 37 started triple antiretroviral combination therapy 9 months after study entry.
¶Patient 29 started triple antiretroviral combination therapy after the second plasmid immunisation.

Table 1: **T-cell data and viral load**

antigen-presenting cells fixed in paraformaldehyde in the presence of interleukin-2 for 14 days[8] and then assayed for cytotoxic effects against autologous target cells.

Target cells were autologous or foreign B-LCLs or cell line T2 (HLA A2)[21] infected with HIV-1/MuLV or vaccinia vectors expressing the HIV-1 genes MVA-*nef*, VVTG-*rev*, VVTG-*tat*. Uninfected cells and cells infected with wild-type vaccinia (MVA-WT) were used as control targets. MHC-class-I mismatched B-cell target controls were done for all patients except patient 37.

CD8 lymphocytes were depleted from isolated peripheral-blood mononuclear cells by means of immunomagnetic beads coated with antibodies to CD8 (Dynal, Oslo, Norway). Depletion of CD8+ cells exceeded 90%.

Cytolytic activity was measured in 4 h ^{51}Cr release assays at various effector:target ratios,[6] applying all tests for non-specific lysis.[22] The controls were assayed in parallel at the same time. The proportion of specific lysis was calculated by subtraction of the percentage lysis of uninfected or MVA-WT infected targets from the percentage lysis of antigen-expressing targets. CTL assays were considered positive if the lysis of antigen-expressing targets exceeded that of control targets by at least 10%. HIV-1 p24 antigen was detected in all culture supernatants from HIV-1/MuLV-infected targets. Vaccinia-specific CTL assessments served as a control for specificity. All patients had had vaccinia vaccination in childhood. The frequency of HIV-1-specific CTL precursors was estimated by limiting dilution analysis.[23]

Lymphocyte proliferation

Lymphocyte proliferation assays[24] were done in triplicate with: Nef or Tat proteins from HIV-1 Bru derived from *Escherichia coli* or Rev protein of HIV-1 LAI derived from a baculovirus-lepidopteran cell system; or control antigens (baculovirus in lepidopteran cells and/or human lung-cell extracts); and medium.[16] The proteins and the control antigens were used at concentrations of 1 µg/mL and 10 µg/mL. After 6 days of incubation, the cells were pulsed with ^{3}H-thymidine and the stimulation index of lymphocyte DNA incorporation was calculated as the mean of experimental counts/min divided by the mean of medium counts/min. Stimulation indices of at least three are conventionally considered positive.[24–26]

Results

Patient follow-up

Table 1 presents all nine patients with their CD4 and CD8 counts and viral loads at days 0 and 194. All nine patients were monitored for 18 months after entry to the plasmid study. None of the patients had developed an AIDS-related complication by 18 months. One patient, immunised with *tat* cDNA, developed local erythema once at the site of injection; no other clinical or laboratory signs of toxicity were noted. Patient 7 was on antiretroviral combination therapy (zidovudine plus didanosine), at entry into the present study in 1996, and has since also started triple antiretroviral combination therapy. Patient 29 started with zidovudine plus lamivudine plus indinavir therapy after the second plasmid injection; this probably influenced

Patient	DNA immunogen	Time*	Highest CTLp frequency†	Vaccinia-specific CTL	T-cell proliferation‡
39	*nef* cDNA	Before	0	Negative	
		After	113	Negative	4/5 (5, 4, 5, 4)
7	*nef* cDNA	Before	1	Positive	
		After	166	Negative	3/4 (6, 9, 43)
3	*nef* cDNA	Before	0	Positive	
		After	78	Negative	3/5 (6, 24, 36)
25	*rev* cDNA	Before	2	Positive	
		After	344	Positive	2/5 (6, 5)
34	*rev* cDNA	Before	0	Negative	
		After	31	Negative	4/5 (181, 212, 18, 8)
37	*rev* cDNA	Before	0	Positive	
		After	16	Negative	0/5
6	*tat* cDNA	Before	4	Positive	
		After	104	Negative	3/5 (4, 5, 4)
29	*tat* cDNA	Before	2	Negative	
		After	46	Negative	3/3 (8, 26, 8)
12	*tat* cDNA	Before	0	Positive	
		After	37	Negative	2/5 (10, 4)

*Analysis done before (day 0) and after DNA vaccination (≥14 days after the third immunisation).
†Frequency of CTL precursors estimated by maximum likelihood method. Results are shown as CTL precursors/10^6 peripheral-blood mononuclear cells, describing the two sampling events before immunisation and five events after immunisation.
‡Results are shown as number of positive assays/total number of assays after first immunisation, stimulation indices from positive assays are shown in parentheses. Medium values used for stimulation-index calculation ranged from 74 to 2146 counts/min (mean 547 cpm), in each case the actual value was used for stimulation-index calculation.

Table 2: **Summary of cell-mediated immune responses before and after DNA immunisation with HIV-1 regulatory genes**

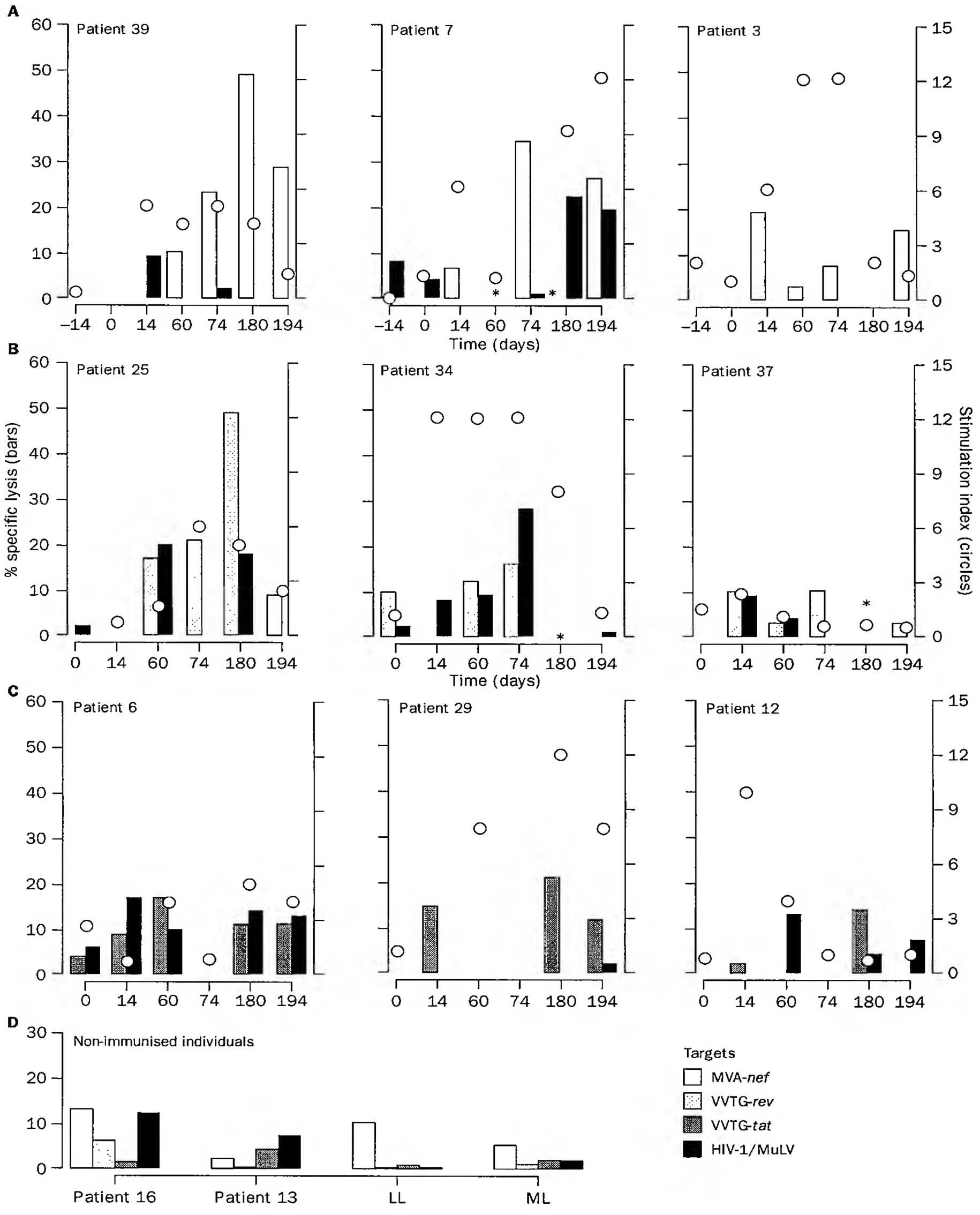

Figure 1: **Induction of cellular immune responses to HIV-1-DNA vaccine with *nef* cDNA (A), *rev* cDNA (B), or *tat* cDNA (C) in symptom-free HIV-1-positive patients and CTL activity (D) in non-immunised symptom-free individuals infected with HIV-1 (16 and 13) and uninfected individuals (LL and ML)**
*CTL not done. Effector:target ratios were 50:1 except for (A).

the CD4 count and viral load. Two patients (3 and 37) started triple combination therapy (zidovudine+lamivudine+indinavir) after completion of the vaccine study. The remaining five patients were still naïve to antiretroviral therapy at the end of the study.

Cytotoxic T-lymphocyte induction

Figure 1 shows CTL activities of stimulated peripheral-blood mononuclear cells from patients immunised with *nef*, *rev*, and *tat* cDNA, respectively, and from non-immunised individuals. Three to eight CTL assays (a mean of four assays) with the target cells defined in figure 1 were done in individual patients before the start of immunisation. CTL assays were negative before DNA vaccination in all nine patients. With *nef*, cytolysis was quite clear; all *nef* studies are therefore shown at effector:target ratios of 6:1, while for *rev* and *tat* they are shown at effector:target ratios of 50:1. The right axis of figure 1 shows the specific proliferative response as the stimulation of indices up to 12 (higher values are shown in table 2). CTL activity in non-immunised symptom-free HIV-1 infected individuals (patients 16 and 13) or uninfected participants (LL and ML) are shown at effector:target ratios of 50:1 (figure 1D).

All three individuals immunised with cDNA *nef* (figure 1A) mounted a substantial and specific cytotoxicity: in patient 39, CTL activity was found from day 74, with an apparently higher response at day 180; patient 7 showed Nef-specific CTL from day 54 and patient 3 from day 14. In patient 3 the response to Nef-expressing target cells was low and variable.

Of the three individuals immunised with cDNA *rev* (figure 1B), one (patient 37) was a non-responder. For patient 25, cytotoxic activity was detected from day 60, with a maximum observed at day 180; after the third immunisation, however, CTL activity decreased (day 194). Patient 34 showed positive cytotoxic responses from day 60 and the highest response at day 74. Patients 25 and 34 were considered to have a transient CTL response.

After *tat* cDNA immunisation, low HIV-1-specific cytotoxic activity appeared in all three patients (figure 1C). In patient 6 specific cytotoxic activity ranged from 10% to 20%. Patients 29 and 12 had CTL responses from days 14 and 60, respectively (considered transient in patient 12).

In the non-immunised HIV-1 infected or non-infected individuals, no CTL response or a low CTL response was detected (figure 1D). A series of CTL assays from these four people was done, with similarly low responses over 9 months. Nef-specific CTL is common in individuals infected with HIV-1.[4]

Three types of specificity controls were done. MHC dependence: MHC-class-1-mismatched B-cell targets were used as controls for all immunised patients, except number 37. They were not recognised in the CTL assays. Origin of effector cells: depletion of CD8+ cells from the effector cell population was accompanied by a considerable decrease in HIV-1-specific lysis, indicating that DNA vaccination induced CD8+ CTL. These studies were done for each antigen in three patients (figure 2). Viral specificity: the vaccinia-specific CTL responses after HIV-1-DNA immunisation were assessed at every sampling: the CTL reactivities were generally low (0–27%), which is to be expected since restimulation with vaccinia had not been done and 20–35 years had elapsed since the vaccinia vaccination. No increased vaccinia-specific CTL reactivities were noted (table 2).

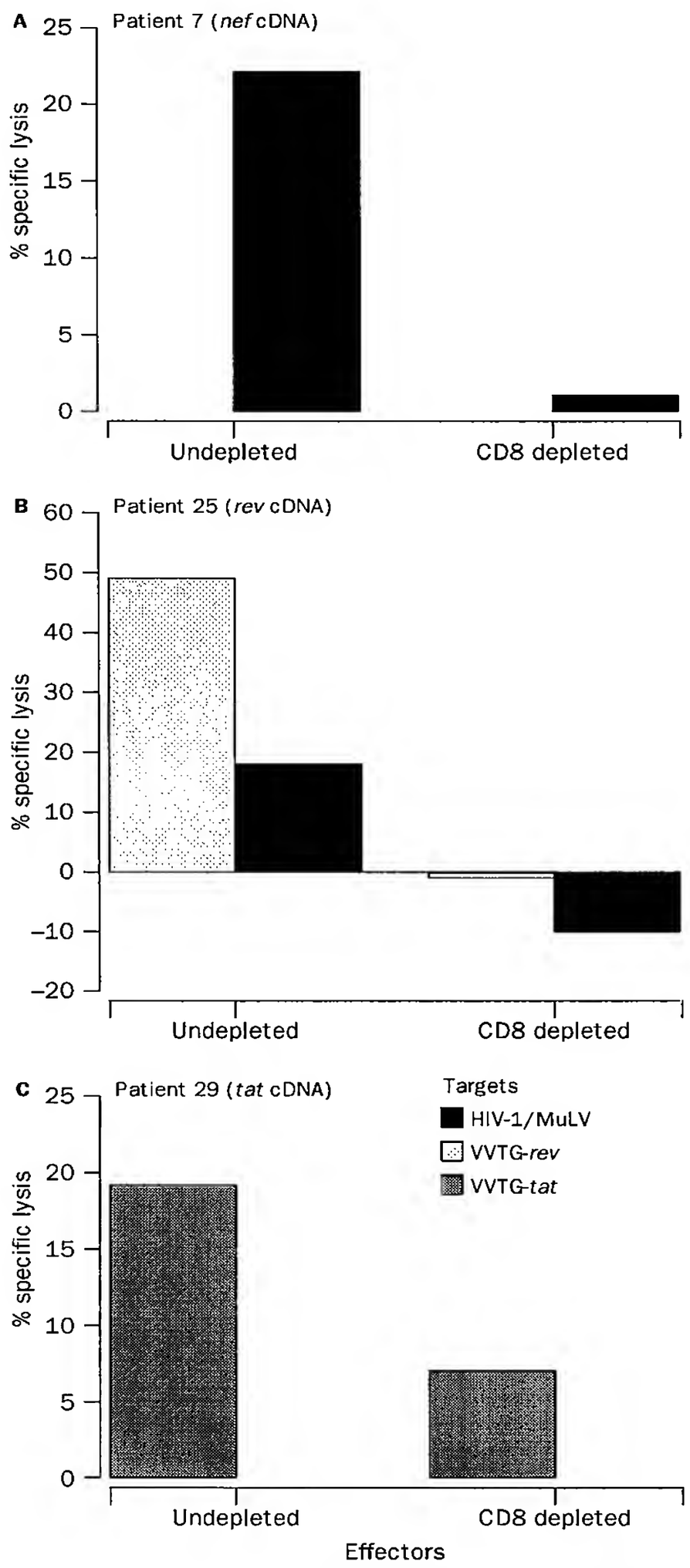

Figure 2: **CTL activity at day 180 of undepleted and CD8-depleted PBMCs against autologous B-cell targets from (A) patient 7, (B) patient 25, (C) patient 29**

CTL=cytotoxic T lymphocyte; PBMC=peripheral blood mononuclear cell. Effector:target ratios were 6:1, 50:1, and 50:1 for patients 7, 25, and 29, respectively.

In summary, endurable HIV-1-specific CTL reactivity was noted in five immunised individuals; three of the remaining patients had transient responses and one did not react (*rev* cDNA immunised). No CTL reactivity was noted with HLA-mismatched target cells. Target cells infected with vaccinia virus carrying the relevant gene mostly displayed a higher percentage lysis than did the HIV-1/MuLV-infected target cells (figure 1).

CTL precursor frequencies

The frequency of effector cells can be estimated from limitation of dilution of the cell population that mediates the cytotoxicity. These estimates correlated well with the CTL results (table 2). A high frequency of CTL precursors was detected for target cells infected with MVA-*nef* (patients 39 and 7); the lymphocytes of patient 3 were assayed against HIV-1/MuLV infected targets. For targets infected with VVTG-*rev*, a high concentration of CTL precursors was detected in patient 25; in the other two patients, the frequency ranged from 16 against HIV-1/MuLV infected targets (in the non-responsive patient 37) to 31/10^6 peripheral-blood mononuclear cells against target cells infected with VVTG-*rev* (patient 34). The frequency of CTL precursors against target cells that express the *tat* gene was highest in patient 6; in patients 29 and 12, the highest CTL precursor frequencies were likewise observed against target cells infected with VVTG-*tat*.

Lymphocyte proliferative responses

Antigen-specific T-cell proliferation indicates T-helper cell activity, usually of CD4+ origin.[24] Proliferatim results are shown together with the cytotoxicity responses in figure 1 and table 2. After the first *nef* cDNA immunisation, lymphocyte populations of all three patients responded to Nef antigen and the proliferative response was detectable in 10 of 14 samples taken after *nef* immunisation. Positive proliferative activity to the Rev antigen was found from day 74 in patient 25; both proliferative and CTL responses decreased after the third immunisation. Strong proliferation to Rev was detected in patient 34, starting at day 14. In patient 37, poor proliferation to Rev coincided with low CTL responses. Tat-specific proliferation was detected in 8 of 13 samples taken after *tat*-immunisation. A correlation between increased cytolytic activities and antigen-specific proliferative responses was observed at several, but not all, time points.

Discussion

The induction of immune responses specific to HIV-1 by a DNA vaccine has not, to our knowledge, been reported in human beings. Our results show that vaccination with plasmid DNA that express the HIV-1 regulatory genes *nef*, *rev*, and *tat* induced HIV-1-specific cellular responses in eight of nine symptom-free HIV-1-infected patients. Immunisation was consistently accompanied by an increased pool of antigen-specific precursor CTLs, which indicates that even immunodeficient individuals have a capacity to respond to immunisation by forming new HIV-1-specific memory cells. Antibody induction, usually at low magnitude, to defined epitopes of the respective proteins was also seen (data not shown).

The patients were selected for having no antibody reactivities or low antibody reactivities to the HIV-1 regulatory proteins. In populations of HIV-1-infected individuals, 50–70% have detectable Nef antibody and/or CTL.[4,27] Our selection criterion was intended to enable us to measure the specific immune responses. The lytic activity detected after immunisation was therefore considered to be associated with the DNA immunisation. It seems reasonable to suppose that even when circulating effector CTLs are already present at low concentration, elevated concentrations of memory cells are also induced by the DNA vaccination.

The transience of the responses in three patients might reflect a subsequent loss of memory T-cells after immunisation. The turnover of lymphocytes of both naïve CD45RA+ and memory CD45RO+ cells is high in HIV-1 infection.[28] Immunisation-induced T-cell proliferative memory against glycoprotein 160 of HIV-1 persists for only a few months.[25] The present finding that the cytolytic precursor cells can be increased by up to a hundred-fold suggests that repeated immunisations might be capable of inducing the relevant immunities in all infected individuals. To achieve persistent responses it may be necessary both to use larger doses or more immunisations and to reduce the viral burden.

Nef is one of the first proteins to be produced in HIV-1-infected cells.[29] CTLs occur naturally in HIV-1 infection, and have been related indirectly to an improved prognosis.[30] In our study, CTL response varied between patients. In the three individuals immunised with *nef* cDNA, a response was detectable at most time points. A CTL response against the Nef protein may prove to be an advantage because several regions of this molecule are conserved between different HIV-1 isolates, Nef peptides are well presented in infected cells, and are also highly immunogenic.[31] In non-infected experimental animals, DNA immunisation induced primary Nef CTL activity.[18] In our already infected individuals, it is more likely that we boosted a pre-existing but low CTL response.

Both Rev and Tat proteins are essential for virus replication.[32] Immunisation with plasmids expressing the HIV-1 *rev* gene induced CTL activity in two patients, while a moderate cytolytic activity was detected in patients immunised with *tat* cDNA. Other studies have shown that CTL responses against Rev are less frequent and that Tat is seldom recognised by CTL in HIV-1-infected individuals.[27,33] Low concentrations of Rev-specific and Tat-specific CTL have been associated with rapid progression to AIDS.[34]

The CTLs induced by DNA vaccination were shown to be specific for the respective HIV-1 proteins. The use of cold target inhibition[22] reduced the background lysis to vaccinia-specific CTLs which was prevalent in some patients. Vaccinia also served as an unrelated virus to which no increased CTLs activity was shown during HIV-1 immunisation. The CTLs were MHC class-I restricted and characterised as being mainly of CD8+ origin. It has been shown, however, that in HIV-1 infection both CD4+ and natural killer cells may lyse infected cells.[35]

The data presented here show that CTLs induced by DNA immunisation lyse autologous cells infected with HIV-1 by the HIV/MuLV pseudovirus or by vaccinia carrying *nef*, *rev*, or *tat* genes. The cells infected by HIV/MuLV pseudotype virus present all viral peptides simultaneously and may better represent the natural situation. They were, however, usually lysed less efficiently than when each HIV-1 gene was expressed alone by vaccinia carrying *nef*, *rev*, or *tat*. This finding suggests that in-vitro CTL assays with B-cells presenting single antigenic peptides of a microbe may overestimate the in-vivo efficacy of lysis of target cells.

The DNA vaccination was done to assess the feasibility of inducing immune reactivities in human beings. The study was open-label, making it possible to analyse the immunogenicity of the individual HIV-DNA constructs. But it also has such drawbacks as a small number of individuals, no blinded controls, and a selected group of patients. The chosen participants may have had

histocompatibility antigens that were unsuitable for response. For Nef this is unlikely because at least HLA A2 (represented by two individuals) are presumably able to respond to Nef peptides. The situation as regards Rev and Tat is not known. Our data provide no evidence of a decreased viral load. Immunisation might be of benefit if combined with the highly active antiretroviral therapy that is now available.

This study supports the feasibility of the use of genetic vaccination to induce new immune responses in human beings. To achieve full immunity, any prophylactic or therapeutic vaccine would no doubt have to include additional—preferably all—HIV-1 associated genes. A combination of DNA constructs that encode the HIV-1 regulatory genes might induce a more complete immune response to the early proteins in individuals who are already infected. These findings may also contribute to a prophylactic vaccination model in non-infected individuals.

Contributors

Sandra Calarota contributed to the execution of the study and she had the main responsibility for the data analysis and writing of the paper. She did the technical work together with Siv Nordlund who redefined the CTL assay. Jorma Hinkula participated in the study design and advised on data analysis and interpretation. The calculation of CTL precursor frequencies was done by Ann-Charlotte Leandersson who also assisted with the laboratory work. Göran Bratt and Eric Sandström were responsible for clinical care of the patients, for sample collection, and provided clinical data. Britta Wahren created the idea for the study, obtained the necessary permissions, supervised the laboratory study, and made detailed manuscript comments. All authors contributed to writing the paper.

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EXHIBIT

AH

Protection of chimpanzees from high-dose heterologous HIV-1 challenge by DNA vaccination

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Novel approaches for the generation of more effective vaccines for HIV-1 are of significant importance. In this report we analyze the immunogenicity and efficacy of an HIV-1 DNA vaccine encoding *env*, *rev* and *gag/pol* in a chimpanzee model system. The immunized animals developed specific cellular and humoral immune responses. Animals were challenged with a heterologous chimpanzee titered stock of HIV-1 SF2 virus and followed for 48 weeks after challenge. Polymerase chain reaction coupled with reverse transcription (RT-PCR) results indicated infection in the control animal, whereas those animals vaccinated with the DNA constructs were protected from the establishment of infection. These studies serve as an important benchmark for the use of DNA vaccine technology for the production of protective immune responses.

Efficacy and safety concerns remain in the field of HIV vaccine development despite extensive laboratory immunological analysis and animal testing. Although no single measure of immunity is predictive of *in vivo* control of viral replication, there is mounting evidence that an efficacious vaccine against HIV-1 will need to induce both cellular and humoral immune responses to control infection. In the majority of cases, the natural immune response generated against HIV-1 is incapable of preventing the eventual onset of AIDS. However, a minority of patients, termed nonprogressors, tend to share a similar immunological phenotype that includes strong antiviral cellular as well as humoral immune responses[1]. Patients mounting strong gp160-specific cytotoxic T lymphocyte (CTL) responses have shown a rapid reduction of acute viremia and antigenemia, and a high level of CTL activity has been associated with long-term survival[2,3]. In contrast, primary viremia and antigenemia were poorly controlled in patients in whom virus-specific CTL activity was low or undetectable. Furthermore, a definite CTL response has been observed in Gambian sex workers who remain seronegative as well as PCR negative for HIV-1 despite repeated exposure to HIV-1 (ref. 4, 5). High levels of antibodies, most notably neutralizing and cross-reactive neutralizing antibodies have also been observed in the serum of long-term survivors[2]. Stronger serological responses have been observed in mothers who do not transmit HIV-1 to their babies[6–9]. In general, stronger and broader immune responses have been associated with delayed disease progression and are likely to have a positive impact on limiting replication, although the ability of the immune response to affect viral replication in infected individuals remains controversial.

Recently, several groups have reported on the ability of the novel vaccine technology, designated genetic vaccination, nucleic acid vaccination or DNA vaccination to induce immune responses *in vivo*. Injection of cDNA expression cassettes results in *in vivo* expression of the encoded proteins[10–12] with the concomitant development of specific cellular and humoral immune responses directed against the encoded antigen(s)[13–17]. The endogenous production of antigen by the host cell transcriptional machinery mimics aspects of live attenuated vaccines without the associated risk of potential pathogenic replication. Protective responses have been reported in several murine disease models using this technology[18–21]. Humoral and cellular responses have been induced to HIV-1 and SIV antigens through various applications of this technology in macaques[22–24] as well as mice[22,25–27]. However, the ability of this technology to induce protective responses after infection in higher nonhuman primate models has never been established[28].

Chimpanzees (*Pan troglodytes*) provide a particularly relevant model for the evaluation of vaccines against HIV-1. The chimpanzee and human immune systems share many common features including antigenic cross-reactivity of divergent

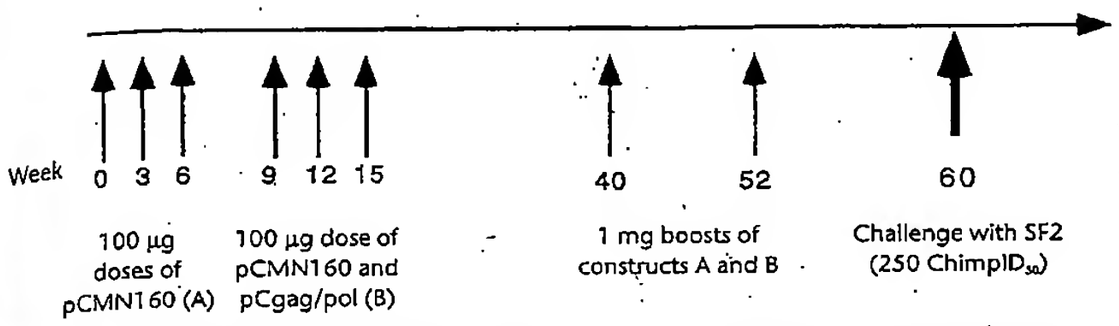

Fig. 1 Schematic of immunization protocol for chimpanzees 19, 83, 94 and 92.

major histocompatibility complex (MHC) antigens as well as T-cell receptor antigens, molecularly and biologically conserved cytokines and costimulatory molecules. In addition, HIV-1 infection of chimpanzees is similar to human infection, as virus can be isolated from peripheral blood mononuclear cells (PBMCs) despite the presence of HIV-1-specific neutralizing antibodies and possibly CTLs. It is noteworthy that HIV-1 isolates derived from infectious human samples that are known to be pathogenic in the natural host can establish persistent infection in challenged animals. Unlike disease progression after HIV-1 infection of humans, disease progression has been observed in a limited number of chimpanzees to date. Analysis of the immune responses induced by DNA plasmid vaccines in naive chimpanzees has importance for understanding the role of this technology in prophylactic vaccine development. For these reasons, we sought to investigate the ability of DNA plasmids to affect HIV-1 replication in the chimpanzee model.

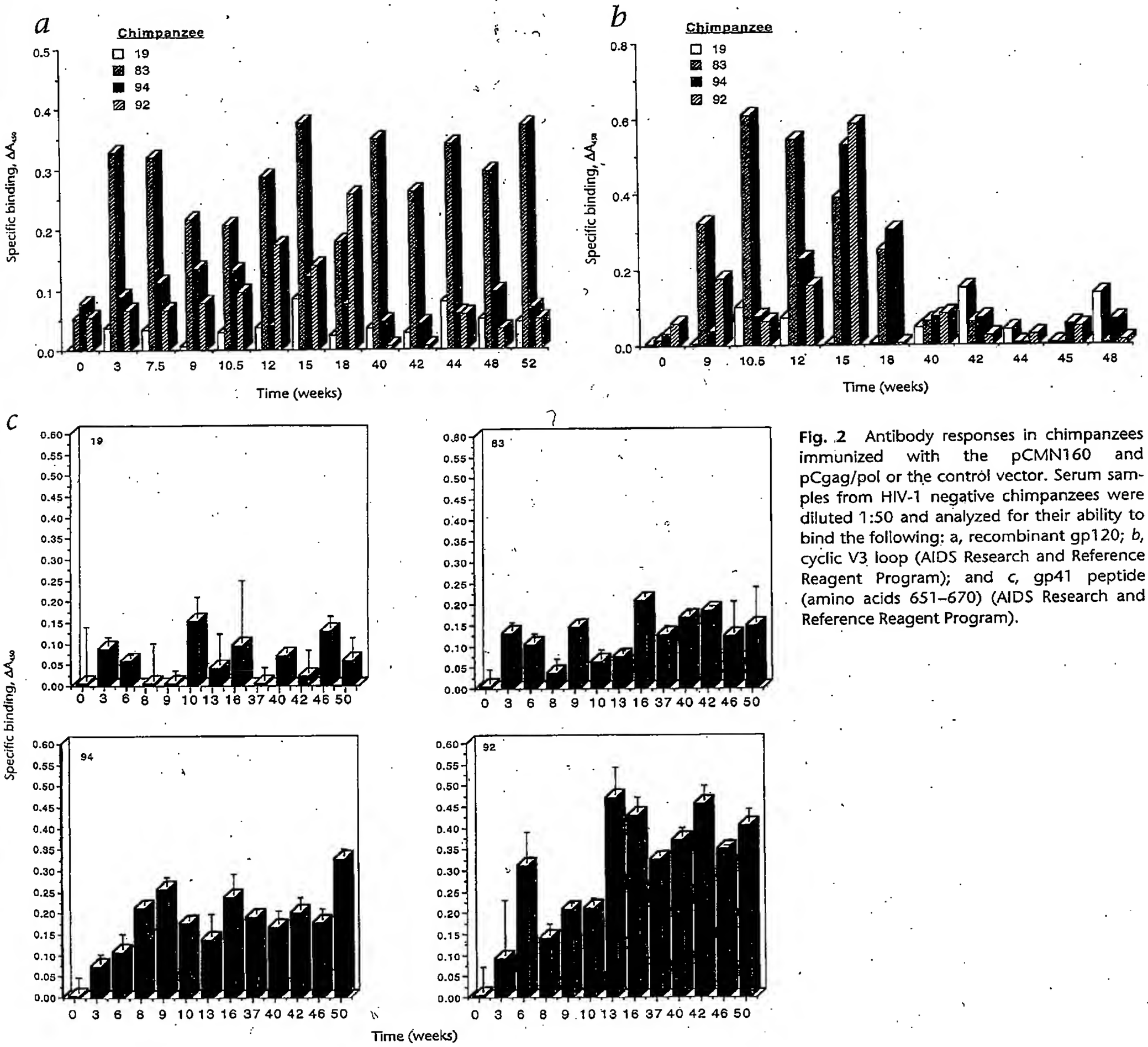

Fig. 2 Antibody responses in chimpanzees immunized with the pCMN160 and pCgag/pol or the control vector. Serum samples from HIV-1 negative chimpanzees were diluted 1:50 and analyzed for their ability to bind the following: a, recombinant gp120; *b*, cyclic V3 loop (AIDS Research and Reference Reagent Program); and *c*, gp41 peptide (amino acids 651–670) (AIDS Research and Reference Reagent Program).

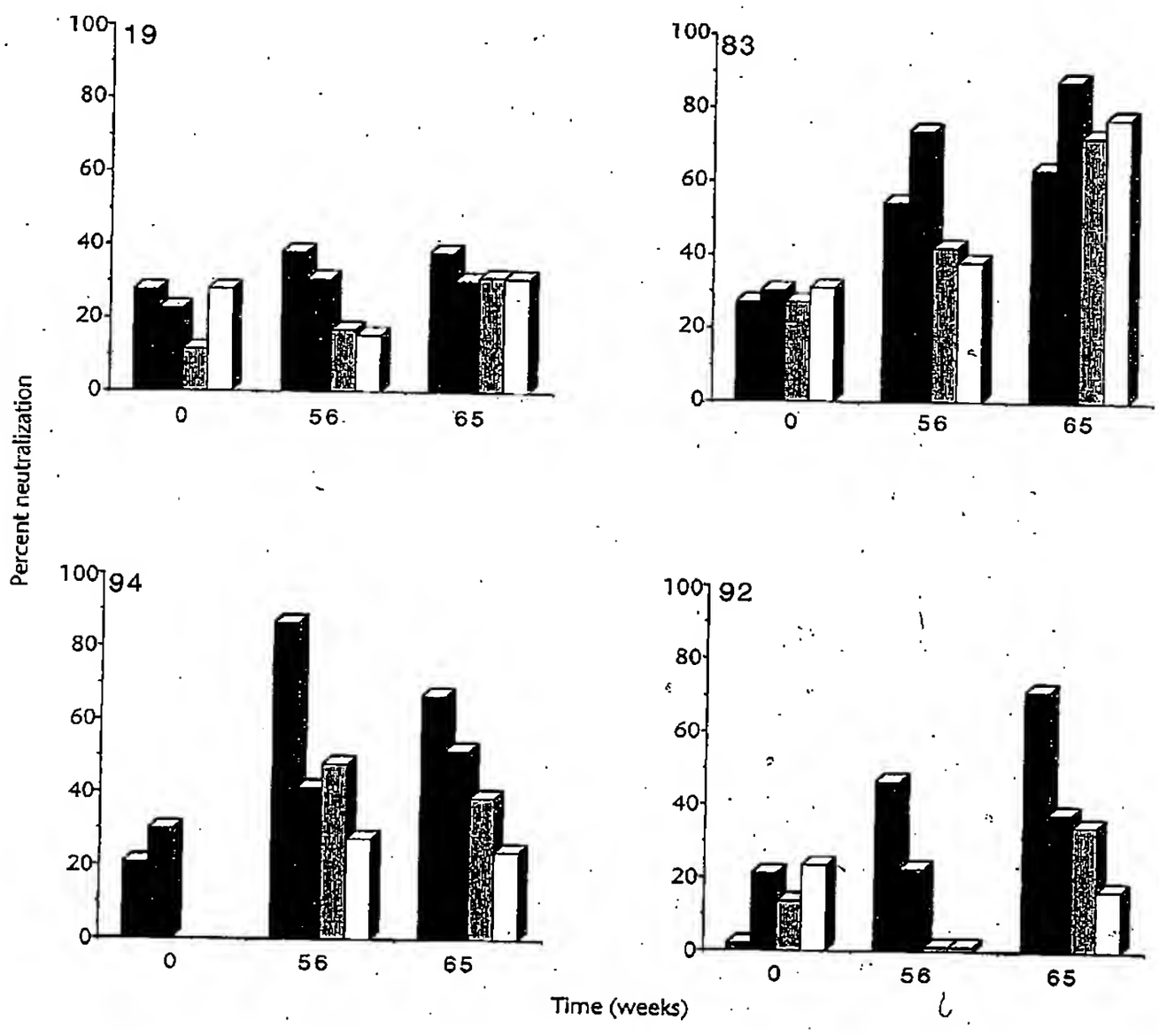

Fig. 3 *In vitro* neutralization of HIV-1 MN. MT2 cells were infected with 100 $TCID_{50}$. Serum at indicated weeks from immunized chimpanzees was diluted 1:20 (black bar), 1:40 (dark gray bar), 1:80 (light gray bar) and 1:160 (white bar). Standard deviations were within 7%.

Serum binding to HIV-1 proteins and peptides

We immunized three chimpanzees (two males and one female) as indicated in Fig. 1. A fourth male chimpanzee was immunized with equivalent doses of an identically prepared control vector lacking the HIV-1 gene insert. Both pre- and post-immunization serum samples from the immunized and control chimpanzees were diluted, and binding reactivity to recombinant envelope protein and a number of HIV-1 envelope-derived peptides was measured. The three immunized experimental chimpanzees developed different antibody profiles against recombinant gp120 as well as the HIV-1 MN V3 loop (Fig. 2, *a* and *b*). In addition, chimpanzee 83 demonstrated antibody responses to several peptides at a dilution of 1:50 (data not shown), including peptides spanning amino acids 421–440, 431–450 and 471–490, whereas chimpanzees 94 and 92 responded to the peptide spanning amino acids 471–490. Furthermore, chimpanzees 94 and 92 demonstrated serological responses to a peptide spanning amino acids 651 to 670 (Fig. 2*c*). This region of gp41 is of interest, as a broadly neutralizing human monoclonal antibody has been characterized that maps to amino acids 651 to 670 (ref. 29). Low-titer antibody responses to core protein p55 were only detected in the serum of chimpanzee 92 (data not shown). In murine studies, 100% of the pCgag/pol vaccinated animals developed strong cellular responses to Gag/Pol targets, but the majority of animals exhibited low-titer antibody responses to Gag antigens by enzyme-linked immunosorbent assay (ELISA) (J.D.B. *et al.*, unpublished). Therefore, vaccination of chimpanzees with HIV-1 DNA constructs induced significant anti-Env antibody responses, but low anti-Gag antibody responses.

Neutralization activity of serum

The antibodies against HIV-1 in these DNA vaccinated animals demonstrated neutralizing activity. Serum from chimpanzee 83, which had exhibited a high serological response to gp120, also demonstrated consistent *in vitro* neutralizing activity against HIV-1 MN at dilutions of up to 1:160. Serum from chimpanzee 94 and 92 also inhibited infection by homologous strains of HIV-1, although at lower neutralization titers (up of 1:20) (Fig. 3). Serum from the animal vaccinated with the vector control failed to neutralize the HIV-1 strain MN virus stock at any dilution.

Cytotoxic T-lymphocyte response

We evaluated CTL responses to Gag/Pol- or Env-expressing targets using immortalized EBV-transformed autologous cell lines. We observed induction of CTL activity against Env targets in bulk PBMCs in chimpanzee 94 at multiple time points following vaccination. The other two vaccinated chimpanzees (83 and 92) failed to demonstrate CTL activity against targets expressing the Env proteins (Fig. 4*a*). In addition, both chimpanzees 83 and 94 developed specific CTL responses to targets expressing the HIV-1 Gag/Pol proteins (Fig. 4*b*) although 94 exhibited a significantly more sustained response. The CTL responses in the control animal, 19, were negligible to either Env- or Gag/Pol- expressing targets.

Viral challenge

Chimpanzee 83, which exhibited the most consistent neutralizing antibody profile, chimpanzee 94, which exhibited the most consistent CTL profile, and the control chimpanzee (19) were challenged with a heterologous, high-dose (250 $ChimpID_{50}$) stock of HIV-1 SF2. Chimpanzee 92 was used as a negative control. Polymerase chain reaction coupled with reverse transcriptase (RT-PCR) (sensitivity of 50 copies/ml[30,31]) was used to assess viremia and was repeated in triplicate on three separate occasions for each sample (Table 1). As expected, the control animal became infected within 2 weeks of challenge and has remained positive by RT-PCR for the 48 weeks following challenge. In con-

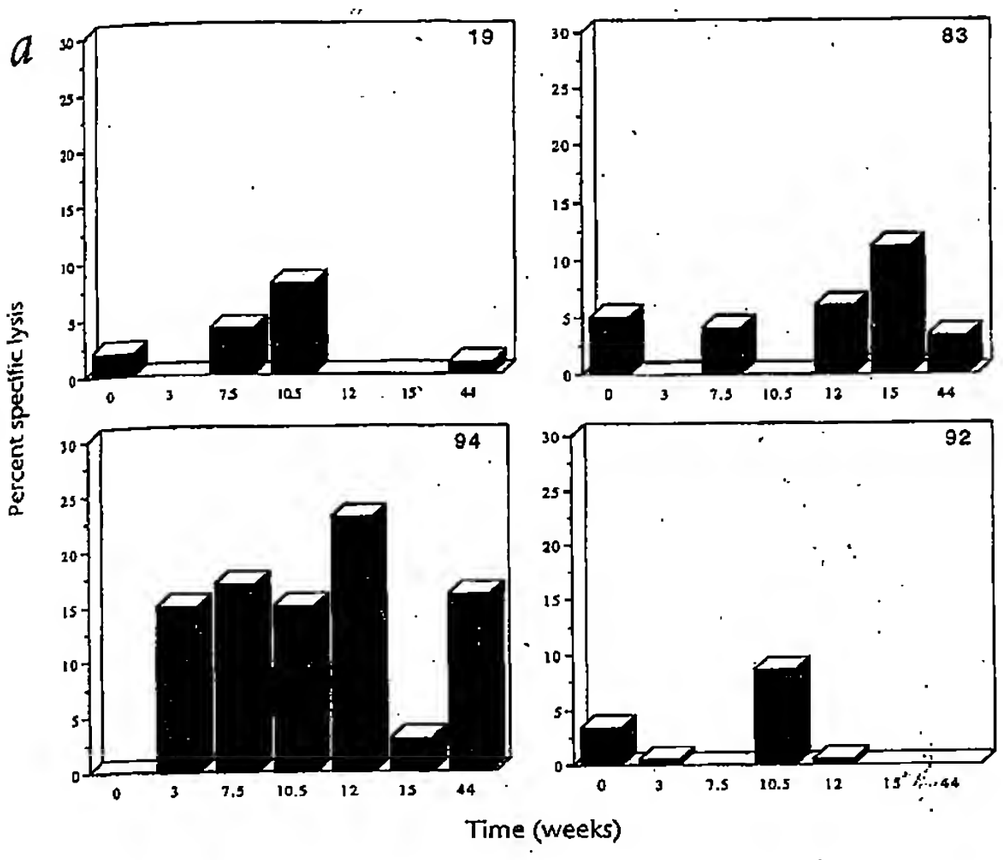

Fig. 4 Cytotoxic T-lymphocyte assay. Percent specific lysis of targets expressing *a*, HIV-1 envelope protein; and *b*, HIV-1 Gag/Pol proteins. Specific lysis is the percent lysis above background lysis of a negative control. Standard deviations were within 7%.

trast, chimpanzees 83 and 94 exhibited a drastically different RT-PCR profile following challenge. We observed RT-PCR positivity in chimpanzee 83 at a single time point (week 6) following challenge. This animal has remained negative as assessed by RT-PCR through week 48 of the study. Similarly chimpanzee 94 was RT-PCR positive at a single time point (week 8), with no virus detectable during the remainder of the study. Samples from week 6 and 8 were analyzed using the Chiron clinical branched-chain assay (sensitivity 500 copies/ml). The results demonstrated that animal 83 and 94 were both negative for weeks 6 and 8, whereas the plasma from the control animal (19) had 10^4 particles/ml on both weeks 6 and 8. Additionally, inguinal lymph node biopsies were taken at week 22 and subjected to DNA PCR analysis as described[32]. The tissue from animal 19 was positive, whereas both 83 and 94, as well as animal 92, were negative. Both humoral and cellular responses were examined post challenge, and viral load was analyzed. Neither immunized chimpanzee 83 or 94 showed boosting in CTLs or antibodies following challenge. CTLs were not detected in the control animal following challenge; western blot analysis was, however, positive. These data support the ability of this vaccination technique to protect chimpanzees from challenge with HIV-1.

CD28 levels following HIV-1 challenge

Changes in T-cell functions and subsets have been described as a consequence of HIV-1 infection[33,34]. In particular, downregulation of $CD28^+$ on $CD8^+$ T cells has been associated with progressive infection[35]. $CD28^+$ is expressed on normal $CD4^+$ T cells and most $CD8^+$ T cells and is upregulated through binding of either the B7.1 or B7.2 ligands, which are presented to T cells along with MHC antigen complexes on antigen-presenting cells. This interaction provides a critical costimulatory signal for T-cell activation. Recent studies support the particular importance of the $CD28^+$ ligand in HIV immunopathology[36]. $CD28^+$ levels were evaluated in both infected and naive control animals (Table 2). The $CD28^+$ profiles observed in these control chimpanzees duplicated the results observed in humans: $CD28^+$ expression decreased in the infected animals. We next examined the $CD28^+$ expression of the experimental chimpanzee PBMCs following challenge (Table 2). Fluorescence-activated cell sorting (FACS) analysis of PBMCs isolated from chimpanzee 19 revealed a $CD28^+$ profile similar to that reported in humans infected with HIV-1. Specifically, significantly lower levels of $CD28^+$ expression were measured coincident with the animal's infection status. In contrast, PBMCs of chimpanzees 83 and 94 demonstrated CD28+ expression analogous to individuals seronegative for HIV-1 (ref. 37). These data further support the viral load observations.

All animals appeared to be in good health throughout the study as assessed by body weights and clinical observations. Furthermore, there was a lack of redness or tenderness observed in any of the animals at the site of injection, consistent with previous studies[24].

Discussion

Of the traditional approaches to vaccination against pathogenic human viruses only live attenuated virus preparations activate both arms of the immune system in a manner similar to that caused by natural infection. However, disease may occur after administration of attenuated vaccines because of viral mutation and reversion to a pathogenic isolate. With these concerns in mind, novel technologies such as DNA vaccination have been examined as viable strategies for the prevention of infection with HIV-1 and other pathogenic human viruses. Indeed, several groups have reported on the induction of specific cellular and humoral immune responses directed against vaccine-encoded antigen(s)[13–17]. Protection has been reported in several murine studies after DNA vaccination[18–21]. In the case of HIV, humoral and cellular responses have been induced in macaques[22–24] as well as mice[22,25–27]. However, before this report, DNA vaccines had never been shown to induce protective responses in the chimpanzee model[28].

In the current study, PBMCs isolated from chimpanzee 94 induced both anti-Env and anti-Gag/Pol CTL activity. Serum from the same animal had measurable levels of antibodies to gp160 as well as low-level neutralizing activity against homologous virus. Chimpanzee 83 demonstrated anti-Gag/Pol CTL activity and consistently high antibody reactivity to HIV-1 envelope, as well as neutralizing antibodies to homologous HIV-1. Chimpanzee 92 failed to demonstrate specific CTL activity above background

Table 1 RT-PCR results from chimpanzees challenged with 250 $ChimpID_{50}$ of HIV-1 strain SF2

Weeks post challenge	Chimpanzee 19[a]	83[b]	94[b]	92[b]
−8	−	−	−	−
−6	−	−	−	−
−4	−	−	−	−
−2	−	−	−	−
0	−	−	−	−
0.5	−	−	−	−
1	−	−	−	−
2	+	−	−	−
4	+	−	−	−
6	+	+	−	−
8	+	−	+	−
10	+	−	−	−
12	+	−	−	−
14	+	−	−	−
16	+	−	−	−
18	+	−	−	−
22	+	−	−	−
26	+	−	−	−
30	+	−	−	−
32	+	−	−	−
36	+	−	−	−
40	+	−	−	−
44	+	−	−	−
48	+	−	−	−
Lymph node biopsy (DNA PCR) Week 22	+	−	−	−

Each sample was tested and evaluated three times by RT-PCR. In addition plasma was tested by Chiron's branched-chain DNA assay. Chimpanzee 83 and 94 by this assay were negative at weeks 6 and 8 as well as at other tested time points. However, chimpanzee 19 demonstrated >10^4 viral particles /ml^3 of plasma at the same time points. [a]Immunized with control vector. [b]Immunized with vectors expressing HIV-1 Env and Gag/Pol.

levels of 10% at any time, but antibodies to HIV-1 envelope and neutralizing antibodies to homologous HIV-1 were measurable in the serum. Although specific anti-HIV responses were induced in both protected animals, no clear correlates of protection could be determined. Because chimps are outbred, they have broad humoral and cellular immune responses, similar to those of humans. Nonetheless, widely different profiles of immune responses resulted in protection from high-dose challenge, suggesting that protection may be attainable through a variety of immune mechanisms either alone or in combination. Previous reports[38,39] have strongly suggested that limiting viral replication at the outset of infection is likely to have a dramatic impact on disease progression in an infected individual. At a minimum, the present study demonstrates the effect of this apparently well tolerated approach to control viral replication *in vivo* and to interrupt the establishment of persistent infection. The further evaluation of this technology for the production of immunogenic DNA expression cassettes to control HIV-1 replication is worth cautious consideration.

Methods

Animals. The study was performed in the spirit of the Good Laboratory Practice regulations and standard operating procedures of Coulston Foundation White Sands Research Center. Furthermore, the study was conducted in accordance with requirements as specifically stated in Section 3.81 of the Animal Welfare Act (9CFR, Ch. 1).

Plasmids. pCMN160 is an expression cassette coding for the envelope and Rev proteins of HIV-1 strain MN. These genes are under the control of the CMV promoter, followed by a polyadenylation region. pCgag/pol is an expression cassette coding for the Gag/Pol proteins of HIV-1 strain IIIB. Plasmids were formulated as previously described[24]. Samples were taken every 1.5 weeks to week 24 at which point the animals were monitored every 2 weeks.

Antibody analysis. A recombinant envelope and a subset of peptides spanning possibly important regions of the envelope protein were analyzed by ELISA. ELISA analysis was performed by modifications of previously described methods[6]. Briefly, recombinant gp120 based on the MN sequence (purchased from Immununodiagnostics, Bedford, MA) was resuspended in 1× PBS to a concentration of 0.5 µg/ml. The gp120 preparation (50 µl, 25 ng) was incubated in each of the wells overnight at 4 °C. For analysis of peptides a concentration of 10 µg/ml in a volume of 50 µl was used. Plates were then rinsed with washing buffer (0.45% NaCl in deionized water containing 0.05% Tween 20) and blocked with blocking buffer (5% nonfat dry milk in PBS + 1% BSA + 0.05% Tween 20) for 2 hours at 37 °C. Serum samples were then diluted in dilution buffer (5% nonfat dry milk in PBS + 0.05% Tween 20) at the appropriate dilutions and incubated in duplicate or triplicate in recombinant protein coated plates for 1 h at 37 °C , washed and then incubated for 1 h at 37 °C with a goat anti-human Ig–horseradish peroxidase conjugate (Sigma Chemical Co.) diluted in dilution buffer at the concentration suggested by the manufacturer. After extensive washing the plates were developed with 3,3',5,5'-tetramethylbenzidine dihydrochloride substrate (100 µg/ml), the reaction was stopped with 2 M H_2SO_4 and color development was quantified at 450 nm. Two human HIV-1 seronegative serum samples were used as controls, and BSA coated wells were used as negative binding control wells in these assays. Specific binding (absorbance at 450 nm) was calculated by subtracting A_{450} values from serum samples bound to BSA (that is, control) from A_{450} values from serum samples bound to gp120; that is, experimental wells (A_{450} experimental – A_{450} control).

Neutralization assay. The ability of serum to neutralize viral infection *in vitro* was assessed according to described methods[40]. Supernatant, 50 µl containing 100 $TCID_{50}$ of HIV-1/MN, was preincubated with 50 µl of serial dilutions of experimental or control chimp serum and added to 3 × 10^4 MT-2 target cells (100 µl). As an internal control we used chimpanzee's positive and negative serum and a mixture of four serum samples from HIV-1-positive and -negative donors. The infection of cells was determined by the presence of syncitia after 48 h of incubation. Neutralization titers above 50% were scored as positive. The percent of neutralization was determined using the following formula:

100% – Number of experimental syncytia (cells + virus + serum)/ Number of control syncytia (cells + virus) × 100%

Cytotoxic T-lymphocyte assay. A standard 5-h ^{51}Cr release CTL assay was performed on PBMCs from the inoculated and control chimpanzees. The preparation of stimulators and effectors has been previously described in detail[24]. Immortalized lymphoblastoid cell lines (LCLs) were established by infecting PBMCs with Epstein-Barr virus. These cell lines were then used as stimulators. Briefly, LCLs were infected overnight with a recombinant vaccinia virus (VMN462) that expressed gp160 from HIV-1 strain MN. Before use, the infected cells were fixed with 0.1% gluteraldehyde and blocked with a 0.1 mM glutamine solution. The fixed cells were incubated with the

Table 2 Expression of CD28 on the cell surface

HIV-1-positive controls						HIV-1-negative control			Challenged animals		
1	2	3	4	5	6	7	8	9	19	83	94
26.6	31.7	15.5	33.4	30.9	35.7	53.3	61.0	65.7	41.5	67.0	64.6

Fresh PBMCs were prepared by standard Ficoll-Hypaque centrifugation and washed extensively. Washed cells were analyzed by FACS for determination of CD28 levels using the well characterized 9.3 monoclonal antibody (PharMingen) as described in the Methods section. The PBMCs were taken from HIV-1-infected chimpanzees or uninfected chimpanzees or were collected at 20 weeks post challenge in the challenged animal study.

freshly isolated PBMCs (effectors) for stimulation. After 3 days of stimulation another batch of LCLs were incubated overnight with VMN462. These cells were labeled with ^{51}Cr and used as target cells. Cells incubated with the a control recombinant vaccinia were used as targets to provide background levels of lysis.

Viral challenge stock. The SF2 viral challenge stock was titered in chimpanzees *in vivo* and was the generous gift of S.M. Nigida (National Cancer Institute).

Reverse transcriptase-PCR. *RNA preparation.* RNA was prepared in accordance with manufacturer's protocol (Biotecx Laboratories, Friendswood, TX) using the "Ultra-spec 3" RNA isolation system with a few internal modifications. Briefly, serum (300 µl) was mixed with 1500 µl extraction buffer (Biotecx, BL-27200) followed by the addition of 200 µl of chloroform and incubated at 4 °C for 5 min. Proteins were then removed by centrifuging at 12,000*g* (4 °C) for 15 min. From this, 800 µl (4/5) of the aqueous layer was transferred to a new 2.0 ml Eppendorf tube containing 1 µl (5 µg/µl) of glycogen (Ambion, Austin, TX) as a carrier, and the RNA was precipitated with a final concentration of 0.5 M ammonium acetate (ABI, Columbia, MD) and equal volume of isopropanol to aqueous solution and stored overnight at –21 °C. Precipitated RNA is collected at 14,000*g* (4 °C) for 15 min, and the remaining pellet is washed with 75% ethanol, pelleted (same) for 5 min, vacuum dried, and resuspended in 20 µl of diethyl pyrocarbonate (DEPC)-treated dH_2O containing RNase inhibitor (1 U/µl, Perkin-Elmer, Norwalk, CT). All RNA were stored at –80 °C.

Reverse transcription reaction. Synthesis of cDNA and ensuing PCR amplification(s) were made using the Perkin-Elmer Cetus DNA Thermal Cycler 480, as well as internal optimization of the GeneAmp RNA PCR kit reagents as described[31]. cDNA synthesis used HIV-1 extracted RNA (1.5 µl of 20 µl) and the following reaction preparation from the Perkin-Elmer RNA PCR kit to obtain 2.5 mM $MgCl_2$, 1× PCR II buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 mM dNTPs, 1 U/µl RNase inhibitor, and 2.5 U/µl MMLV-RT, plus 25 pmol of the HIV-1 specific LTR-*gag* region external antisense primer AV13 5'–CTGCGAATCGTTCTAGCTCCCTGCTTGCCC–3' (antisense NT 895-924 of HXB2)[30]. The synthesis was brought to a final volume of 10 µl with sterile H_20 and overlaid with 40 µl of mineral oil (Perkin-Elmer) and a reverse transcription run of 10 min at 23 °C, 60 min at 42 °C, 10 min at 99 °C, and finally quickly chilled on ice.

PCR and nested PCR amplification. To the above HIV-1 cDNA product (10 µl), a 30-µl reaction volume containing 1× PCR II buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 25 pmol external sense primer AV 10 5'–TGTGACTCTGGTAACTAGAGATCCCTCAGA–3' (sense NT 574-603 of HXB2, AC K03455)[30] of the LTR-*gag* region, and dH_20 (sterile) was underlaid and a "hot start" reaction was initiated.

The mixture was run for one cycle of 2 min at 96 °C, followed by 10 min at 80 °C with a 1.25 U/50 µl AmpliTaq DNA polymerase (Perkin-Elmer) in sterile dH_20 (10 µl total) being underlaid 5 min into the latter temperature sequence for a total PCR sample volume of 50 µl before the amplification sequence cycles (below).

Reaction conditions on the 480 DNA Thermal Cycler for the external PCR were as follows: 25 cycles of 1 min at 94 °C, 1.5 min at 70 °C, and 1 min at 72 °C. For the internal PCR (N-PCR below), conditions were 25 cycles of 1 min at 94 °C, 1.5 min at 47 °C, and 1 min at 72 °C. Additionally, in both cases, the reactions were finished off with a 4 min at 72 °C extension and >5 min at 4 °C soak. All PCR products stored at –21 °C. N-PCR was prepared using 1 µl (1/50th) of the RT-PCR product and a master mix from Perkin-Elmer GeneAmp RNA PCR kit reagents for a 1× PCR II buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 1 mM dNTPs, 1.5 mM $MgCl_2$, sterile dH_20, and 1.25 U/50 µl AmpliTaq DNA polymerase, plus 10 pmol of HIV-1 internal LTR-*gag* region primer AV11 5'–TCTAGCAGTGGCGCC–3' (sense NT 628-642 of HXB2), and AV12 5'–GACGCTCT CGCACCC–3' (antisense NT 791-805 of HXB2)[30] for a 50-µl reaction volume. Samples were again overlaid with 40 µl mineral oil and the above N-PCR reaction conditions were initiated. A commercially available HIV-1-positive control plasmid DNA (Perkin-Elmer, N808-0016) was simultaneously tested during PCR and N-PCR reactions using the same external and internal primer sets. As it was DNA, no reverse transcription was performed. All PCR products were analyzed on a 4% agarose/1× TBE gel with a sample load of 10 µl, electrophoresed for 60 min at 150 V, followed by ethidium bromide staining.

FACS analysis. Cells were isolated as described above[24] by Ficoll-Hypaque and washed twice in FACS buffer (1× PBS, 1% BSA, 0.1% NaN_3). The cells were incubated at a concentration of 3 × 10^5 cells with anti-CD28 conjugated antibody (33745X, PharMingen, San Diego, CA) for 1 h at 4 °C in the dark. The cells were washed twice in FACS Buffer and fixed in 0.5 ml 2% Paraformaldehyde. The cells were analyzed on EPICS XL flow cytometer (Coulter Corp., Hialeah, FL).

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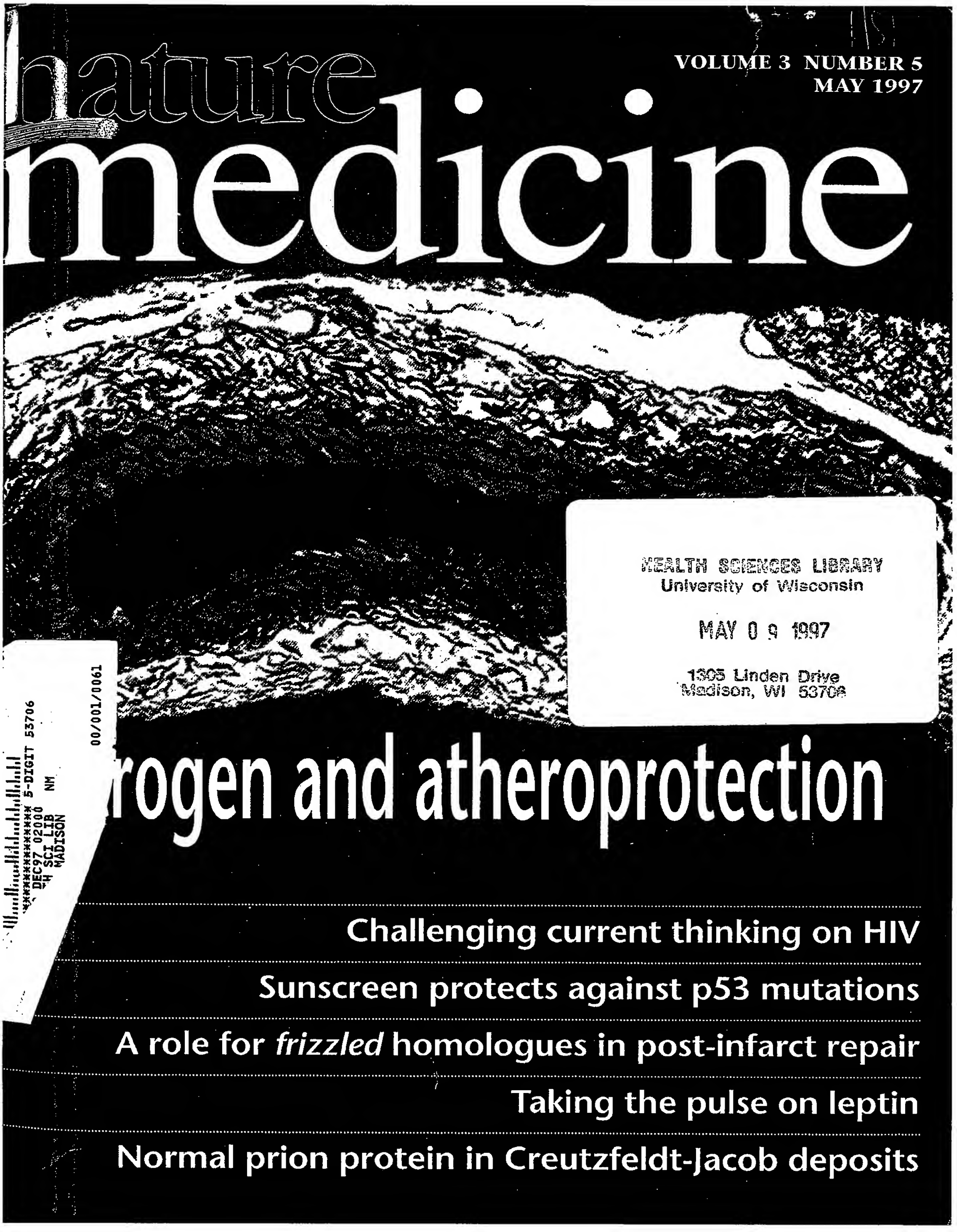

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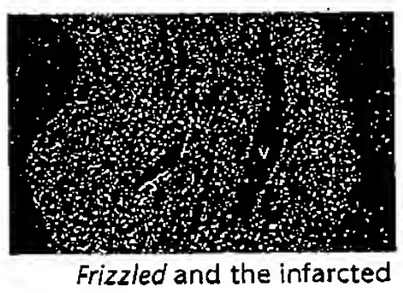

Frizzled and the infarcted heart (page 541).

NOS and bladder dysfunction (page 571).

REVIEW

ARTICLES

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EXHIBIT

AI

DNA vaccines

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Preclinical DNA vaccine development has continued apace during the past year, with the investigation of several new infectious and non-infectious disease targets as well as advances in our understanding of some of the basic immunologic mechanisms, such as effector cells, responsible for conferring protection. The coming year promises to be at least as exciting, as initial human clinical studies have begun.

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Abbreviations

APC	antigen-presenting cell
CEA	carcinoembryonic antigen
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
GM-CSF	granulocyte macrophage-colony stimulating factor
HA	hemagglutinin
HBsAg	hepatitis B surface antigen
IL	interleukin
L1	major capsid protein
NP	nucleoprotein
PCR	polymerase chain reaction

Introduction

DNA vaccines are a recent addition to the armamentarium of potential vaccine technologies and offer promise for the improvement of existing vaccines. Furthermore, targets against which it has been difficult to make vaccines using existing methodology may become feasible if DNA vaccine technology is used instead. DNA vaccines consist of plasmid DNA expression vectors that, when administered to an animal, result in expression of an antigen *in situ* leading to the induction of antigen-specific immunity. The first demonstration of the protective efficacy of a DNA vaccine in an animal model was reported only three years ago [1]. Since then, studies have shown immunogenicity or protective efficacy of DNA vaccines in preclinical (animal) models for a variety of disease targets; and early human clinical studies have been initiated. Clearly, the validation of DNA vaccines awaits the results of such clinical studies and requires continuous evaluation of safety. DNA vaccines, however, offer a number of attractive attributes: their simplicity; the apparent robustness of the technology (a number of effective constructs have been made with off-the-shelf reagents); the breadth of their efficacy and applicability to various pathogens and diseases; and, unlike certain conventional vaccines, their ability to induce vigorous cellular immune responses. This article will review recent publications that expand the potential applications of DNA vaccines or that explore the immunologic mechanisms of protection.

Preclinical efficacy of DNA vaccines

Influenza

The initial demonstration of DNA vaccine efficacy in an animal model was accomplished using the influenza virus [1], which continues to provide a useful system with which to characterize immune responses to DNA vaccines. Influenza is one of the infectious disease targets for a DNA vaccine currently being investigated in human clinical trials. One rationale for efforts to develop an influenza DNA vaccine, despite the availablity of widely utilized existing influenza vaccines, is that current vaccines are effective only in a strain-specific manner. Mutations in circulating influenza virus strains mean that frequent re-evaluation and reformulation of the vaccine is necessary. DNA vaccines offer the advantage of stimulating the generation of cytotoxic T lymphocytes (CTLs) against epitopes from a conserved protein of the virus, such as nucleoprotein (NP), thereby providing cross-strain protection in a mouse model [1]. A study by Donnelly *et al.* [2•] further investigated the immunogenicity and protective efficacy of influenza DNA vaccines and compared them to the licensed conventional vaccines in nonhuman primates and in ferrets (which are considered to be the model of choice for influenza challenge studies because of their suseptibility to human clinical isolates of influenza virus). A combination DNA vaccine containing plasmids encoding both internal and coat proteins of the virus was prepared in a mix intended to mimic the proteins present in the whole virus vaccine. Vaccination of nonhuman primates generated titers of hemagglutination inhibiting antibodies (used as surrogates for neutralizing antibodies) as high as or higher than those generated by the full human doses of whole inactivated and split inactivated virus vaccines, respectively. Ferrets immunized with the DNA vaccine cocktail shed less virus in nasal washings after challenge with a drifted (i.e. antigenically different) strain of virus than did control animals immunized with the analogous commercially licensed whole inactivated virus vaccine. Furthermore, the level of protection induced by the DNA cocktail was statistically indistinguishable from that observed in homologous positive controls; that is, animals immunized with DNA encoding the hemagglutinin (HA) surface glycoprotein of the challenge virus (i.e. homologous virus). This study was significant for several reasons: it was a comparison of a DNA vaccine with a widely utilized licensed clinical vaccine; it was a demonstration of immunogenicity at low doses of DNA in nonhuman primates (10 μg of relevant construct per

dose, given twice; a regimen of two immunizations is customary for administration of the licensed vaccine in naive individuals); it was a demonstration of the efficacy of a cocktail DNA vaccine consisting of a mixture of plasmids encoding several antigens of the same virus; and it was a demonstration of efficacy against a naturally arising strain of virus that was antigenically distinct from the vaccination strain. The inability of the current vaccines to provide protection against such antigenically distinct viruses is one of their chief limitations which may thus be overcome by DNA vaccines.

Malaria

Another model in which a combination DNA vaccine was recently employed is malaria. It had been shown previously that DNA encoding the circumsporozoite protein of *Plasmodium yoelii* could induce CD8+ CTLs and confer protective immunity in mice [3]. The breadth of this protection was recently shown to be genetically restricted, however, although the inclusion of a DNA construct encoding another antigen (PyHEP17) circumvented this limitation by providing epitopes for another haplotype [4••]. Furthermore, Doolan *et al.* [4••] demonstrated that interferon-γ and nitric oxide play key roles in protection. These results suggest that, in addition to induction of specific antibodies and CTLs, DNA vaccines may stimulate other less specific immune mechanisms to combat intracellular pathogens.

Other viral and bacterial disease models

Also important for bringing DNA vaccine technology closer to clinical application was the continued expansion of the breadth of preclinical infectious and noninfectious disease targets successfully tested in animal models. These included additional viral targets, as well as parasitic diseases, bacterial diseases, and cancer (against which the first clinical trials for DNA vaccines were initiated during 1995). Joining the viral disease targets for which immune responses and/or protection had already been demonstrated (such as influenza, bovine herpesvirus, hepatitis B, HIV and rabies) were hepatitis C virus [5,6], herpes simplex virus [7–10], papillomavirus [11], lymphocytic choriomeningitis virus [12–14] and flavivirus [15]. The list of parasitic diseases for which preclinical efficacy has been demonstrated was expanded from malaria [3] and leishmaniasis [16] to include schistosomiasis [17]. These results are important for several reasons. First, hepatitis C represents a disease for which no vaccine currently exists. Vaccine technology that can generate a CTL response might offer an important advantage over certain other types of vaccines because the viral core protein (best targeted by a CTL response rather than an antibody response) is relatively well conserved and because an antibody response alone might be of limited protective efficacy. Second, herpes simplex virus and papillomavirus are sexually transmitted pathogens with very high incidences of causing disease. Furthermore, their collective morbidity and mortality are not simply limited to primary viral infections per se, but include recurrent disease and, importantly, the potential for progression to carcinoma, in the case of papillomavirus. Third, the demonstration of protection in a rabbit model of papillomavirus [11] was significant from the standpoint of the immunobiology of DNA vaccines as well. In that report the DNA vaccine encoded the major capsid protein, L1, against which neutralizing antibodies are directed. When L1 is synthesized in an infected cell the protein is directed to the nucleus where nascent virus is assembled; epitopes against which neutralizing antibodies are directed are conformational in nature. Thus, it was somewhat surprising that a DNA vaccine encoding only L1 was capable of generating a protective antibody response in rabbits, as measured by prevention of condyloma development upon subsequent challenge with rabbit papillomavirus. These results indicate that L1 can attain a native conformation and be transported to a cellular location amenable to the generation of neutralizing antibodies in the absence of other papillomavirus proteins.

It is possible that bacterial diseases were not the initial target for DNA vaccines because a number of protective bacterial antigens are not proteinaceous (e.g. polysaccharides) and because less is known about the protective antigens of bacteria compared with viruses (i.e. those antigens that induce a protective immune response). For at least some bacterial targets, however, not only are proteins key antigens, but the types of cellular responses induced by DNA vaccines may be important elements of an effective vaccine. An example of such a bacterial target is *Mycobacterium tuberculosis*; the cellular responses against its proteins appear to play key roles in protective immunity. Recent work [18;19] has shown that tuberculosis DNA vaccines induce CTL and helper T cell responses of the Th1-like phenotype, as measured *in vitro* upon restimulation of spleen cells with antigen. Such responses have been reported with DNA encoding antigen 85 [18] and hsp65 [19]. Furthermore, protective efficacy conferred by DNA vaccines encoding these single *M. tuberculosis* antigens was comparable to that induced by the clinical Bacille Calmette-Guerin (BCG) vaccine which consists of a whole live organism whose efficacy is quite variable and which can cause disease in immunocompromised individuals.

Toward an understanding of immunologic mechanisms

Tolerance

From an immunologic standpoint, perhaps the most striking recent observations were obtained with hepatitis B DNA vaccines [20•,21•]. Mice transgenic for the surface antigen of hepatitis B (HBsAg) are tolerized by virtue of ontogenetic expression of the antigen. These mice express HBsAg in their hepatocytes and do not develop antibodies or CTLs against the expressed antigen, either spontaneously or after immunization with recombinant HBsAg. When they were immunized with a

plasmid encoding HBsAg, however, they developed both antibodies and CTLs with a concomitant elimination of antigen expression (Davis *et al.*, unpublished data). These results suggest that immunologic tolerance to the antigen in these transgenic animals was broken by this vaccination regimen. Interestingly, the expression of the transgene ceased without any evidence of immune-mediated hepatic damage, suggesting that elimination of HBsAg expression was mediated at a transcriptional or translational level rather than by destruction of antigen-expressing cells.

Increased breadth of immunogenicity

Other studies with HBsAg DNA have shown induction of immune responses in strains of mice that had previously shown little or no responsiveness to the antigen given as proteins. Mice of the H-2^b haplotype (C57BL/6) are considered to be nonresponsive to HBsAg at the CTL level because they do not generate MHC class I-restricted CTLs following immunization with either recombinant HBsAg or recombinant vaccinia virus expressing HBsAg. Vaccination with HBsAg DNA, however, generated both H-$2K^b$- and H-2D ^{b}-restricted CTL responses to HBsAg epitopes [20•]. In mice with low responsiveness to HBsAg at the antibody level, HBsAg DNA was shown to induce immune responses following a single immunization, whereas use of the recombinant HBsAg required two immunizations before immune responses were seen [21•]. While these studies may not indicate that haplotype restriction was broken, they do indicate that antigen processing and/or presentation with DNA vaccines differs quantitatively and/or qualitatively from that found in other modes of vaccination.

Induction of CTLs

Until recently, CTL responses have been induced with DNA vaccines encoding full-size antigens: that is, entire proteins. In many cases this would be desirable to allow antigen processing and determinant selection in an outbred population. In some instances, such as for antigenically variable epitopes, however, it may be advantageous to direct the responses to specific epitopes of a protein. Such epitope-targeted immune responses have been induced using DNA vaccines containing minigenes encoding single CTL epitopes from mutant p53 and HIV gp120 [22]. In these cases it was found that enhanced CTL responses were attained when the peptides were preceded by an endoplasmic reticulum targeting signal sequence. This minigene approach should prove to be useful for induction of epitope-specific responses, especially for subdominant epitopes that may not induce responses in the presence of other more dominant epitopes.

One of the more intriguing unexplained issues regarding DNA vaccines is the nature of induction of CTL responses. The two more common means of administering DNA vaccines, intramuscular injection and particle bombardment, both induce MHC class I restricted CTLs. By the particle bombardment method, cells of the dermis and epidermis are transfected by the direct penetration of the DNA-coated gold beads. Since antigen presenting cells (APCs), such as Langerhans cells, are found in these layers of the skin; CTLs may be induced by direct transfection of these 'professional' APCs. In contrast, intramuscular injection results in the uptake of extracellular DNA by, and antigen expression in, muscle cells (and possibly in other cells, although this has not been documented). The importance of antigen expression by muscle cells in the induction of CTLs is not yet known. Several lines of evidence suggest that muscle cells may play a role. Firstly, while various routes of NP DNA administration can lead to the induction of CTLs, only intramuscular injection conferred substantial CTL-mediated protection from a cross-strain, lethal challenge with influenza virus [23]. Secondly, polymerase chain reaction (PCR) analyses of DNA from several tissues after intramuscular injection indicated that most, if not all, plasmid DNA was localized to the muscle [24•]. Therefore, while it is possible that intramuscular injection of DNA results in the transfection of nonmuscle cells, there is no evidence so far to support this possibility. Thirdly, transfection of nonmuscle cells is not required for the induction of MHC class I restricted CTLs and protective immunity, as demonstrated following transplantation of NP-expressing myoblasts into syngeneic mice [25]. Finally, the induction of CTLs after transplantation was mediated, at least in part, by transfer of antigen from the transplanted muscle cells to APCs of the recipient mice [25]. Further studies are required to delineate the cells responsible for CTL induction; however, taken together, these results suggest that intramuscular injection of DNA results in expression of antigen by muscle cells, but that the induction of CTLs is mediated by nonmuscle APCs, possibly by transfer of antigen from muscle cells to APCs.

Modulation of immune responses

In preclinical studies administration of DNA vaccines is an effective means of inducing antibodies and CTLs. The efficacy of DNA vaccines could be enhanced or modulated through the use of formulations that increase DNA stability or distribution in the muscle, the coexpression of immune molecules that affect the processing of antigens, or through the use of adjuvants that affect the immune responses that are mounted against the expressed antigen. With respect to adjuvants, in recent studies DNA vectors expressing cytokines have been shown to be biologically active *in situ* [26] and to affect immune responses against coexpressed antigens [27,28]. In the latter studies, granulocyte macrophage-colony stimulating factor (GM-CSF) was shown to have a stimulatory effect on both humoral and cellular immune responses to rabies virus glycoprotein [27] and carcinoembryonic antigen (CEA) [28]. Recombinant interleukins (ILs) -7 and -10 have also been used to increase the effectiveness of DNA vaccines in a tumor challenge model [29]. Others have used DNA encoding costimulatory molecules B-7.1 and B-7.2 in an effort to enhance or modulate immune responses

mounted against a coexpressed antigen by potentially providing an additional means of T cell stimulation. Using this approach, increased antibody responses against *M. tuberculosis* hsp65 were seen in a combination DNA vaccine containing B-7.2 DNA [19] and enhanced antibody and antitumor responses were induced by coinjection of DNA plasmids encoding CEA and B-7.1 [28].

Another potentially effective and simple way of enhancing immune responses to DNA vaccines is via an adjuvant effect of the DNA itself. Work over the past five years has shown that certain sequences can induce cytokine secretion and lymphocyte activation [30,31,32•]. Certain CpG motifs in bacterial DNA are particularly stimulatory, whereas similar DNA from other species are not; this is probably due in part to the methylation state of the DNA [32•]. Other CpG motifs can inhibit lymphocyte stimulation [31]. These observations suggest that manipulation of DNA vaccines to contain or avoid these motifs may affect the immunogenicity of antigens expressed by the vector. Preliminary observations have indicated that coinjection of a DNA plasmid (not expressing a protein) with a protein antigen (NP or HBsAg) modulates the isotype profile of the antibodies generated against the antigen, with a shift from an IgG1 predominance after injection of protein alone to an IgG2a predominance after injection of protein and DNA (JB Ulmer, CM DeWitt, RR Deck, MJ Caulfield, MA Liu, unpublished data). The immunomodulatory effect of bacterial DNA was also recently reported in an autoimmune mouse model. The results revealed that New Zealand Black/New Zealand White (NZB/NZW) mice spontaneously develop pathogenic antibodies to DNA and renal disease leading to premature death. Vaccination of pre-autoimmune NZB/NZW mice with bacterial DNA, but not mammalian DNA, accelerated the onset of anti-DNA antibody formation [33]. Unexpectedly, though, this vaccination regimen protected the mice from disease and death [34•], despite the fact that the DNA antibodies induced closely resembled the pathogenic antibodies seen in untreated mice. In addition, vaccination of mice in the advanced stages of disease prolonged their survival. Together with the aforementioned adjuvant properties of bacterial DNA, these results indicate that the composition of a DNA vector may be an important consideration in designing a DNA vaccine.

DNA delivery

Another way in which DNA vaccines could be modified is by the use of a delivery system, such as liposomes or polymers that can compact DNA and enhance cellular uptake, or the inclusion of peptides or proteins that can facilitate intracellular targeting of DNA to the cytoplasm and nucleus. In addition, DNA vaccines may be targeted to specific tissues such as mucosal sites for the induction of mucosal immune responses. To this end, Sizemore *et al.* [35•] have prepared a bacterial vector capable of DNA delivery. The attenuated *Shigella* they developed contains an *asd* (aspartic/semialdehyde dehydrogenase) mutation that does not interfere with the ability of the organism to invade cells but which causes it to burst open inside the cell, thereby releasing expression plasmids into the cytoplasm. Recombinant *Shigella* containing plasmids expressing galactosidase under the control of the human cytomegalovirus (CMV) early promoter and enhancer are not themselves able to express galactosidase but can direct expression of galactosidase in cell cultures, in the guinea pig eye, and in the mouse lung. Mice immunized in this manner produced cellular immune responses and high levels of specific antibodies. HIV and malaria genes have also been expressed in this system. These findings open up the possibility for the relatively inexpensive oral delivery of functional DNA with the potential for manipulation of the local immune system as well as for production of systemic responses (D Sizemore, A Branstrom, J Sadoff, unpublished data).

Expression library immunization

The identification of the protective antigens of a pathogen is a laborious and sometimes problematic process. This is particularly true for protection that requires cellular immunity, since certain types of vaccines (e.g. subunit proteins or whole inactivated viruses) do not generally induce CTLs. Furthermore, testing specific antigens requires that they be available in a purified form. With DNA vaccines, CTLs are readily induced and one needs only to have the gene encoding the antigen. The process of vaccine antigen discovery may be simplified by a recent and exciting application of DNA vaccine technology. Barry *et al.* [36••] developed a method to test mixtures of DNA plasmids containing fragments of the genome of a pathogen for protective efficacy (termed expression library immunization, or ELI). In their example, vaccination with mixtures containing 3000 distinct plasmids from a *Mycoplasma pulmonis* DNA library were shown to confer protection in a mouse challenge model, indicating that at least one of the plasmids encoded a protective antigen. By successive fractionation and testing of these mixtures it may be possible to identify the protective plasmids, although to date such fractionation and identification has not been reported. One of the potential drawbacks of the technique in addition to the potential masking of epitopes by immune interference is that, because fragments of the genome are used, many of the plasmids will not encode a relevant protein. This problem can be overcome by cloning open reading frames into the expression library and, with the burgeoning field of genomics, this sequence information is rapidly becoming available for many pathogens. Such approaches may greatly facilitate the identification of vaccine antigens.

Conclusions

The use of DNA vaccination has grown substantially in the three years since it was first demonstrated that DNA could confer protective immunity. Recent important advances have been made in several areas of DNA vaccines.

These include an expansion of the targets for DNA vaccine development, a greater understanding of some of the underlying mechanisms involved in the induction of immune responses, the beginnings of alternative DNA delivery vehicles that can target mucosal immune sites, the application of DNA vaccine technology to the discovery of protective antigens and the commencement of human clinical trials. Arguably the most significant of these is the latter, since the eventual success of DNA vaccines will be predicated on their effectiveness in humans.

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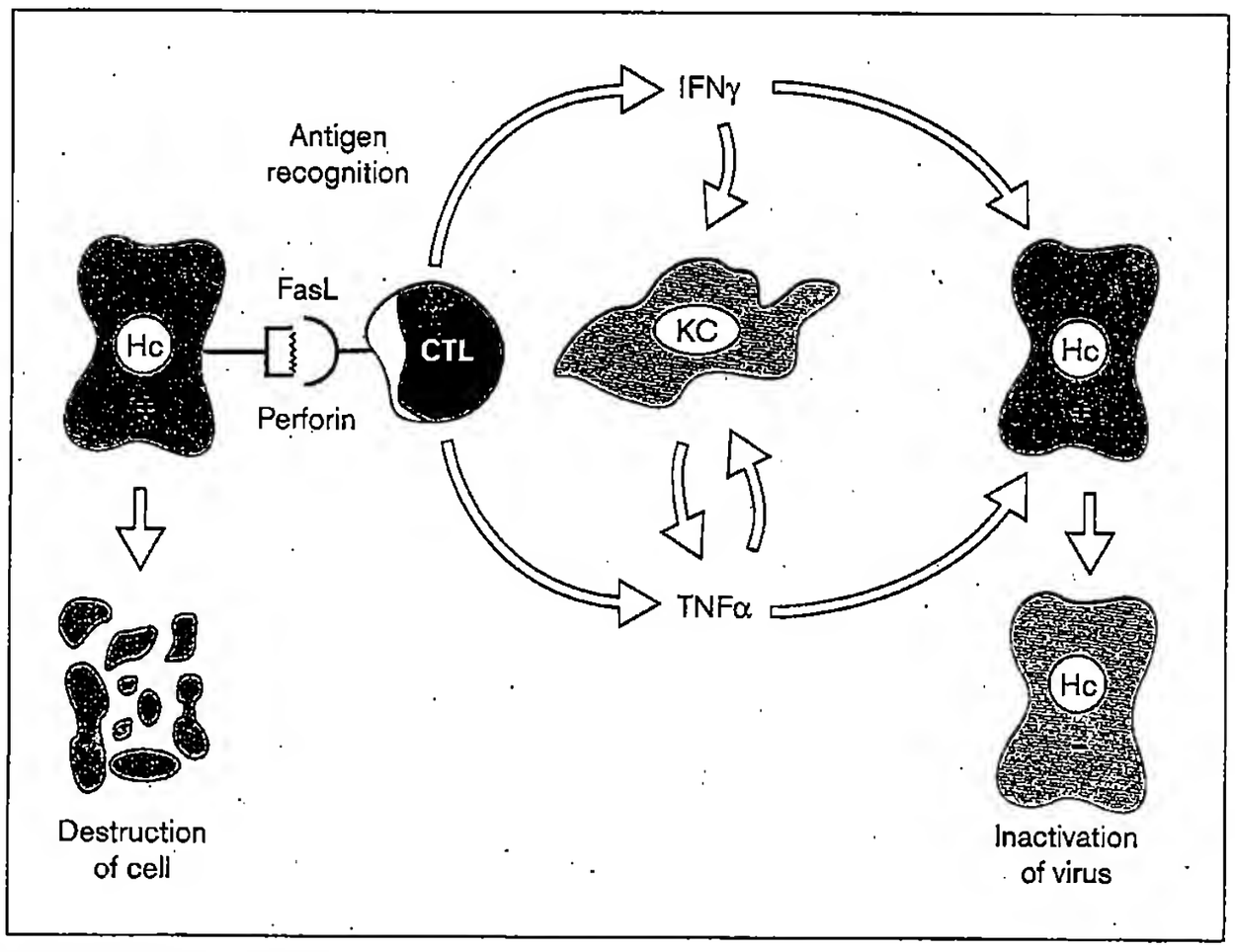

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EXHIBIT

AJ

DNA immunization protects nonhuman primates against rabies virus

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More than 40,000 people die annually from rabies worldwide[1]. Most of these fatalities occur in developing countries, where rabies is endemic, public health resources are inadequate and there is limited access to preventive treatment[2]. Because of the high cost of vaccines derived from cell culture, many countries still use vaccines produced in sheep, goat or suckling mouse brain[3]. The stability and low cost for mass production of DNA vaccines would make them ideal for use in developing countries[4]. To investigate the potential of DNA vaccines for rabies immunization in humans, we vaccinated *Macaca fascicularis* (*Cynomolgus*) monkeys with DNA encoding the glycoprotein of the challenge virus standard rabies virus, or with a human diploid cell vaccine (HDCV). The monkeys then were challenged with a non-passaged rabies virus. DNA or HDCV vaccination elicited comparable primary and anamnestic neutralizing antibody responses. All ten vaccinated monkeys (DNA or HDCV) survived a rabies virus challenge, whereas monkeys vaccinated with only the DNA vector developed rabies. Furthermore, serum samples from DNA- or HDCV-vaccinated monkeys neutralized a global spectrum of rabies virus variants *in vitro*. This study shows that DNA immunization elicits protective immunity in nonhuman primates against lethal challenge with a human viral pathogen of the central nervous system. Our findings indicate that DNA vaccines may have a promising future in human rabies immunization.

Rabies is an almost invariably fatal encephalomyelitis[5]. The World Health Organization estimates that 10–12 million people in developing countries receive one or more doses of rabies vaccine after exposure to the virus. An estimated 5 million people in China and over 1 million people in India begin treatment[1]. Many developing countries continue to use vaccines produced in sheep, goat or suckling mouse brain, with subsequent inactivation of the virus with ultraviolet light or phenol[6,7]. The World Health Organization recommends that vaccines derived from brain tissue be discontinued and replaced with vaccines produced in tissue culture[8]. Although vaccines derived from cultured cells, such as the human diploid cell vaccine (HDCV), are very effective and well tolerated, they are expensive to produce[3].

DNA vaccines offer a new and powerful approach for the generation of needed vaccines[4]. Advantages of DNA vaccines that distinguish them from conventional vaccines are the ease of their construction, their ability to induce a full spectrum of long-lasting humoral and cellular immune responses, and their high temperature stability, which would be a particular advantage for use in tropical areas, where refrigeration is difficult to maintain. Moreover, the low cost for their mass production makes DNA vaccines ideally suited for developing countries. Two important preliminary steps before human clinical investigation begins are to determine the utility of the DNA vaccine in eliciting protective immunity in nonhuman primates and to compare the effectiveness of DNA immunization with that of vaccination with a standard such as the HDCV. Thus, nonhuman primates (*Macaca fascicularis* (*Cynomolgus*) monkeys) were immunized with DNA encoding a rabies virus glycoprotein or with HDCV, and then challenged with a lethal dose of rabies virus.

Thirty days after immunization, a peak antibody titer was detected in the two monkeys that had been vaccinated with HDCV. The antibody titer of the four monkeys immunized with DNA by gene gun was much lower (Figs. 1 and 2). After the peak antibody response was attained at 30 days, the neutralizing antibody titer of the HDCV-immunized monkeys gradually decreased, whereas the antibody titer of the monkeys vaccinated by gene gun increased approximately fourfold by day 60, and then remained fairly constant for 180 days. Rabies neutralizing antibody was not detected in the four monkeys that had been vaccinated intradermally by needle injection with DNA. All monkeys were administered booster vaccinations 190 days after primary immunization (Figs. 1 and 2). Ten days after the booster, the geometric mean neutralizing antibody titer of the HDCV-vaccinated monkeys had increased more than 18-fold, whereas the geometric mean antibody response of the monkeys that had been vaccinated with DNA by gene gun had an even more dramatic increase of 176-fold. For the next 220 days, antibody titers of the monkeys that had been vaccinated with HDCV or DNA using the gene gun gradually decreased to similar levels. In distinct contrast to the anamnestic neutralizing antibody responses of the monkeys vaccinated by gene gun and HDCV, rabies neutralizing antibody remained undetectable in monkeys that had received primary and booster intradermal needle injection vaccinations of DNA. Consequently, at 60 days after booster immunization, the non-responsive monkeys vaccinated by intradermal injection were re-vaccinated, but with 100 μg of DNA delivered intramuscularly. Subsequently, a minimal antibody titer was detected in each of the four monkeys at day 10, increasing to a peak geometric mean titer by day 30 (Figs.1 and 2).

To test the efficacy of the DNA vaccine in comparison with HDCV, the twelve monkeys were challenged with a non-passaged rabies virus isolate from a salivary gland homogenate of a naturally infected rabid dog[9]. At least two human deaths in Texas have been associated with transmission of this virus since

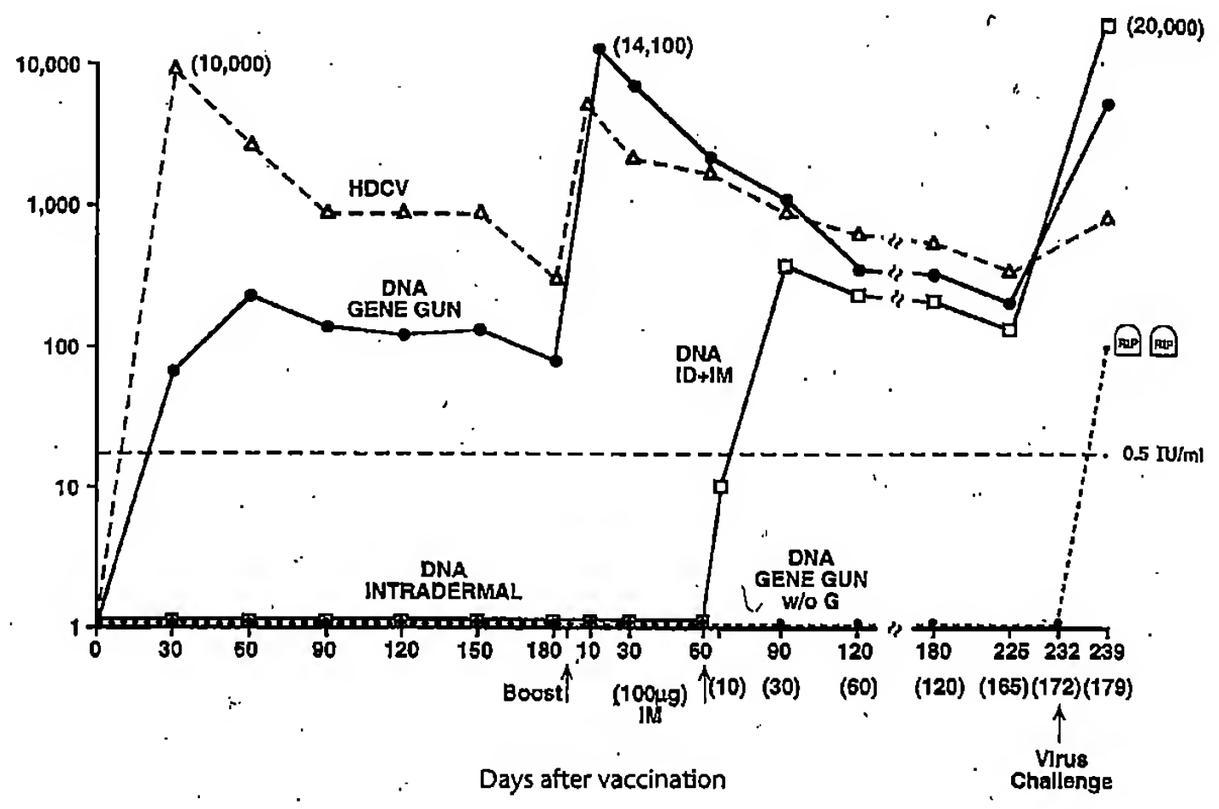

Fig. 1 Geometric mean anti-rabies-virus neutralizing antibody titers in serum after DNA or HDCV vaccination and subsequent rabies virus challenge. DNA encoding glycoprotein administered by intradermal and then intramuscular injection (□) or by gene gun (•—•): Four of four monkeys (100%) protected in each group. HDCV administered by intramuscular injection (△): two of two monkeys (100%) protected. DNA without glycoprotein (vector alone) administered by gene gun (•----•): Zero of two monkeys (0%) protected. Horizontal dashed line, 0.5 IU/ml, is the minimal acceptable human level of antibody titer. Numbers in parenthesis on horizontal axis indicate days after 're-boosting' of monkeys initially immunized intradermally (□). RIP: The two negative-control monkeys that were not protected (•----•) developed CNS signs of rabies and were sedated and killed.

1980. At the time of the challenge, the antibody titer of each vaccinated monkey exceeded the minimal acceptable human level[10] of 0.5 IU/ml (1:40 serum dilution) (Fig. 2). The titer of only one DNA-vaccinated monkey exceeded the titers of the monkeys that had received HDCV. One week after virus challenge, neutralizing antibody titers of the ten vaccinated monkeys had increased considerably. Furthermore, the individual titers of six of eight (75%) DNA-vaccinated monkeys exceeded the titers of the monkeys that had received HDCV (Figs. 1 and 2). At this time, neutralizing antibody was detected for the first time in the two negative control monkeys that had received only the DNA vector (Figs. 1 and 2). On days 10 and 11 after the challenge, the negative control monkeys displayed lethargy, anorexia, cranial nerve deficits, altered phonation and paresis, all abnormal CNS clinical signs associated with rabies. Consequently, they were sedated and killed. Brain impressions of both monkeys were positive for rabies virus antigen as detected by a direct fluorescent antibody test. Six months after the rabies virus challenge the ten vaccinated monkeys were killed. All brain impressions were negative for rabies virus antigen.

Table Worldwide isolates of rabies virus neutralized[a] with serum samples from monkeys vaccinated with DNA or HDCV

Reservoir	Country
Dog	Indonesia
Dog	Thailand
Dog[b]	Laos
Dog[b]	Philippines
Dog	China
Fox	USA-Alaska
Dog	India
Dog	USA-Texas
Dog[b]	Mexico
Laboratory strain (ERA)	USA-Alabama
Laboratory strain (CVS)	France-Pasteur Institute
Dog	Nigeria
Vampire bat	Latin America
Red bat	USA-Florida
Silver-haired bat	USA-Arkansas
Raccoon	USA-Massachusetts
Skunk	USA-Texas
Little brown bat	USA-Montana

[a]In each instance, 100% of the infectivity was neutralized. [b]Virus isolates from humans who had been bitten by rabid dogs and died from rabies after incubation periods of 1, 4 or 6 years. ERA, Evelyn-Rokitnicki-Abelseth; CVS, challenge virus standard.

Recent studies have identified many rabies virus variants associated with different animal reservoirs and geographical areas[11–14]. To determine whether antibody produced in nonhuman primates immunized with a DNA vaccine would neutralize variant viruses that have been isolated worldwide, serum samples obtained before the virus challenge were tested in neutralization assays against 18 different viruses (Table). Serum samples from the DNA- or HDCV-vaccinated monkeys that had been adjusted to contain 0.5 IU/ml of antibody neutralized 100% of the infectivity of each worldwide isolate of virus, as well as the rabies virus laboratory strains, challenge virus standard and Evelyn-Rokitnicki-Abelseth (Table). In contrast, antisera generated by vaccination with the vector alone failed to neutralize any of the viruses (data not shown). These neutralization data, in combination with the protection data, indicate that a DNA vaccine encoding for the glycoprotein of challenge virus standard could protect against a global spectrum of rabies viruses.

The method of DNA vaccination proved to be important parameter in these studies. Rabies neutralizing antibody was not detected in the four monkeys that had been vaccinated intradermally by needle injection with DNA, whereas monkeys that received DNA by gene gun delivery or by a combination of intradermal and intramuscular needle injections produced appropriate antibody responses. The failure of the monkeys to develop appropriate antibody responses after intradermal needle injection vaccination alone was somewhat perplexing, as the same plasmid injected intradermally elicited high levels of neutralizing antibody in rodents[15]. The data demonstrate that immunization results in other species cannot necessarily be used to predict results extrapolated to nonhuman primates. The determination that DNA vaccination does protect nonhuman primates against a considerable rabies virus challenge permits the investigation of additional issues such as the requirement for a booster vaccination, the duration of protection and the possibility of prophylaxis after exposure.

This study indicates that DNA vaccines may have a promising future in human rabies immunization. The elevated neutralizing

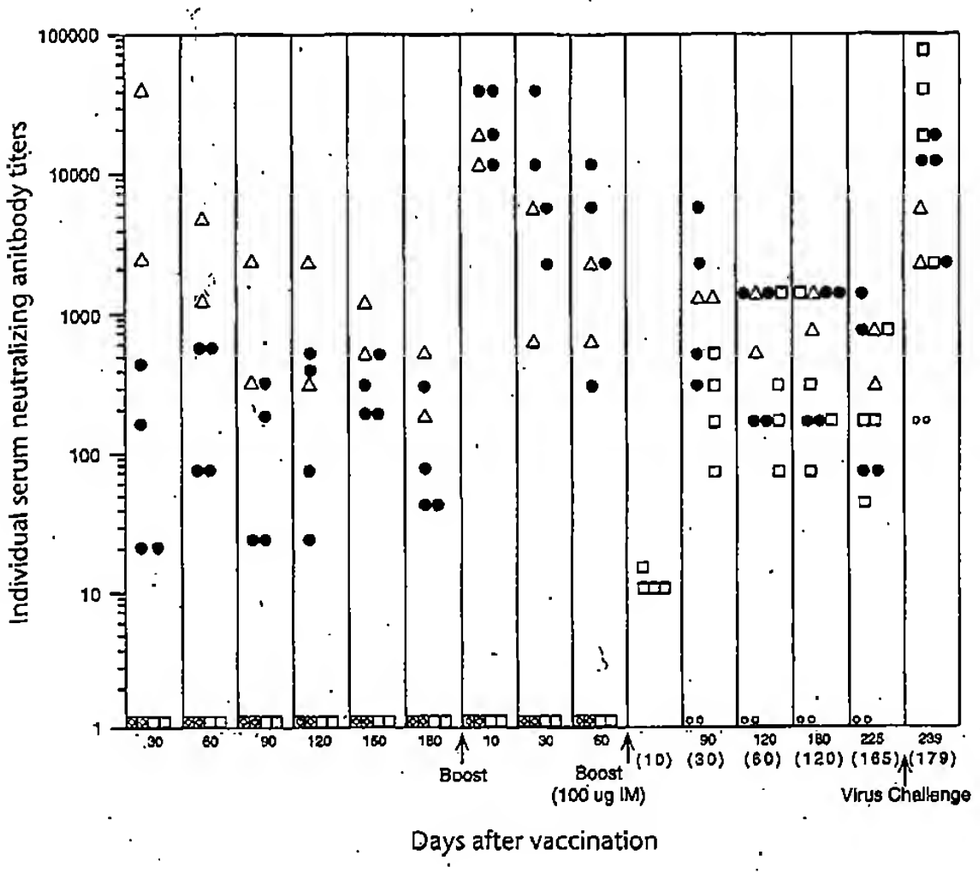

Fig. 2 Individual anti-rabies-virus neutralizing antibody titers in serum after DNA or HDCV vaccination and subsequent rabies virus challenge. HDCV administered by intramuscular injection (△) or DNA encoding glycoprotein administered by gene gun (●). DNA encoding glycoprotein administered intradermally and intramuscularly by needle injection (□). DNA without glycoprotein administered by gene gun (○). Numbers in parenthesis on horizontal axis indicate days after 're-boosting' of monkeys initially immunized intradermally (□).

antibody titers of the DNA-vaccinated monkeys after virus challenge are particularly noteworthy, as neutralizing antibody is the primary source of protection against rabies in the rodent system[16]. DNA vaccines could eliminate immunization with vaccines derived from tissues of the nervous system and could also provide an alternative to the costly HDCV and other cell-culture vaccines, especially for prophylactic purposes. Although nonhuman primates have been used in other DNA vaccination studies[17–20], this DNA vaccine study is the first, to our knowledge, to show complete protection of nonhuman primates against lethal challenge with a primary isolate of a human viral pathogen that invades the immune-privileged CNS.

Methods

Plasmid construction. Construction of the pCMV4 plasmid DNA vaccine encoding the glycoprotein of the challenge virus standard rabies virus has been described[15,21].

Macaca fascicularis (*Cynomolgus*). The *Macaca fascicularis* (*Cynomolgus*) colony at the Rocky Mountain Laboratories (RML) originated with captive monkeys from Mauritius Island. Ten of the experimental monkeys used in this study were wild-caught stock (3 to 8 years in the RML colony), while two were captive-born (from the RML colony). The monkeys in the RML colony are negative for retroviral and herpes B virus antibodies. The monkeys, randomly assigned to experimental groups, ranged in age from 3 to 10 years, with the six females ranging from 3.5 to 4.4 kg in weight and the males, from 4.1 to 9.9 kg in weight.

The monkeys were fed commercial high-protein monkey chow supplemented with fresh fruit and commercial monkey treats. Automatic watering systems provided water *ad libitum*. All monkeys, except the two colony-born males, had been used in previous experimental research involving a benign, self-limiting ocular infection with *Chlamydia trachomatis*. Inoculations and blood samples (collected from the cephalic vein using a 20-guage needle and a 5-ml syringe) were done while the monkeys were sedated with ketamine hyrochloride.

The animal facilities and animal care and use programs at the RML are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and function in accordance with all United States Department of Agriculture, Department of Health and Human Services and National Institutes of Health regulations and standards. The monkeys were housed indoors in artificial light (12 h/12 h dark/light cycle) and housed paired or singly in aluminum barred cages (15.1 square feet floor space × 63 inches high or 6.3 square feet floor space × 32 inches high, respectively). The colony rooms are maintained at 21 °C to 26 °C at 50% humidity. For virus challenge, the monkeys were shipped to the Centers for Disease Control and Prevention, Atlanta, Georgia by a domestic, commercial air freight provider, in full accordance with all United States Department of Agriculture, Department of Health and Human Services and National Institutes of Health regulations and standards. The husbandry of the Centers for Disease Control and Prevention animal facility is similar to that of the RML facility.

Immunizations. Primary immunizations with DNA were done either by the Dermal PowderJect XR gene gun delivery system (PowderJect Vaccines, Madison, Wisconsin) as described[21] or by intradermal needle injection. Four monkeys, each receiving 8 μg of DNA, were included in each group. The monkeys treated by gene gun were vaccinated above the axillary area of each upper arm and above the inguinal area of each upper thigh with 2 μg of DNA in each site. The DNA was coated onto 0.5 mg of gold beads 2.6 μm in diameter, according to the instructions provided by the gene gun manufacturers. The beads were administered with a helium pressure setting of 400 p.s.i. The monkeys vaccinated by intradermal injection were inoculated by needle in the same areas as the monkeys vaccinated by gene gun with an identical amount of DNA suspended in phosphate-buffered saline. Two negative-control monkeys were vaccinated by gene gun with a total of 8 μg of vector DNA only. Two positive-control monkeys received 0.5 ml of HDCV (Pasteur Merieux Serums and Vaccins S.A., Lyon, France) intramuscularly in each triceps muscle. A second vaccination identical to the first was given 7 days later. Booster immunizations identical to the primary vaccinations were given 190 days after primary immunization. The HDCV-vaccinated monkeys did not, however, receive a second booster 7 days after the initial 190-day booster. Sixty days after the 190-day booster immunization, the monkeys injected intradermally by needle were re-boosted, but with 100 μg of DNA delivered intramuscularly in the triceps muscle. Blood was collected at regular intervals and serum samples were tested for anti-rabies-virus neutralizing antibody.

Neutralizing antibody assay. Serum anti-rabies-virus neutralizing antibody titers were established by using the rapid fluorescent focus inhibition test[22] with chicken-embryo-related cells[23] and Evelyn-Rokitnicki-Abelseth virus at a multiplicity of infection of 1. The antibody titers in Fig. 1 are expressed as geometric means for each group of monkeys, whereas individual antibody titers are shown in Fig. 2. Antisera with known IU/ml of rabies virus neutralizing antibody, a rabies hyperimmune mouse serum and the U.S. Standard Human Rabies Immunoglobulin R2 were included as positive controls in all rapid fluorescent focus inhibition tests. An experimental titer of 1:40 was equivalent to 0.5 IU/ml. To test serum samples in neutralization assays against viruses that have been isolated worldwide, monkeys vaccinated with DNA or the HDCV were bled before the virus challenge. Serum samples were diluted to contain 0.5 IU/ml of neutralizing antibody, and then tested in a rapid fluorescent focus inhibition test against each virus that had been adjusted to a multiplicity of infection of 0.1:1.

Rabies virus challenge. The monkeys were challenged with rabies virus at the Centers for Disease Control and Prevention, Atlanta, Georgia. One week before the challenge (225 days after the initial booster; 422 days after primary vaccination), the monkeys were sedated with ketamine hydrochloride (10 mg/kg, injected intramuscularly), and blood samples were obtained to determine anti-rabies-virus neutralizing antibody titers. For the viral challenge, the monkeys were again sedated and then were inoculated in the right and left masseter muscles with 0.5 ml of a 1:5 dilution of a salivary gland homogenate obtained from a rabid dog naturally infected with a coyote rabies virus variant[9]. The viral titer of the stock salivary gland homogenate was $10^{6.5}$ mouse intracranial lethal $dose_{50}$/ 0.03 ml. After the challenge, the monkeys were observed several times daily for abnormal clinical signs associated with rabies. Animals showing abnormal CNS clinical signs associated with rabies were sedated and killed. At necropsy, the brains

were removed and brain impressions were made. The brain impressions were tested for rabies virus antigen by the direct fluorescent antibody test .

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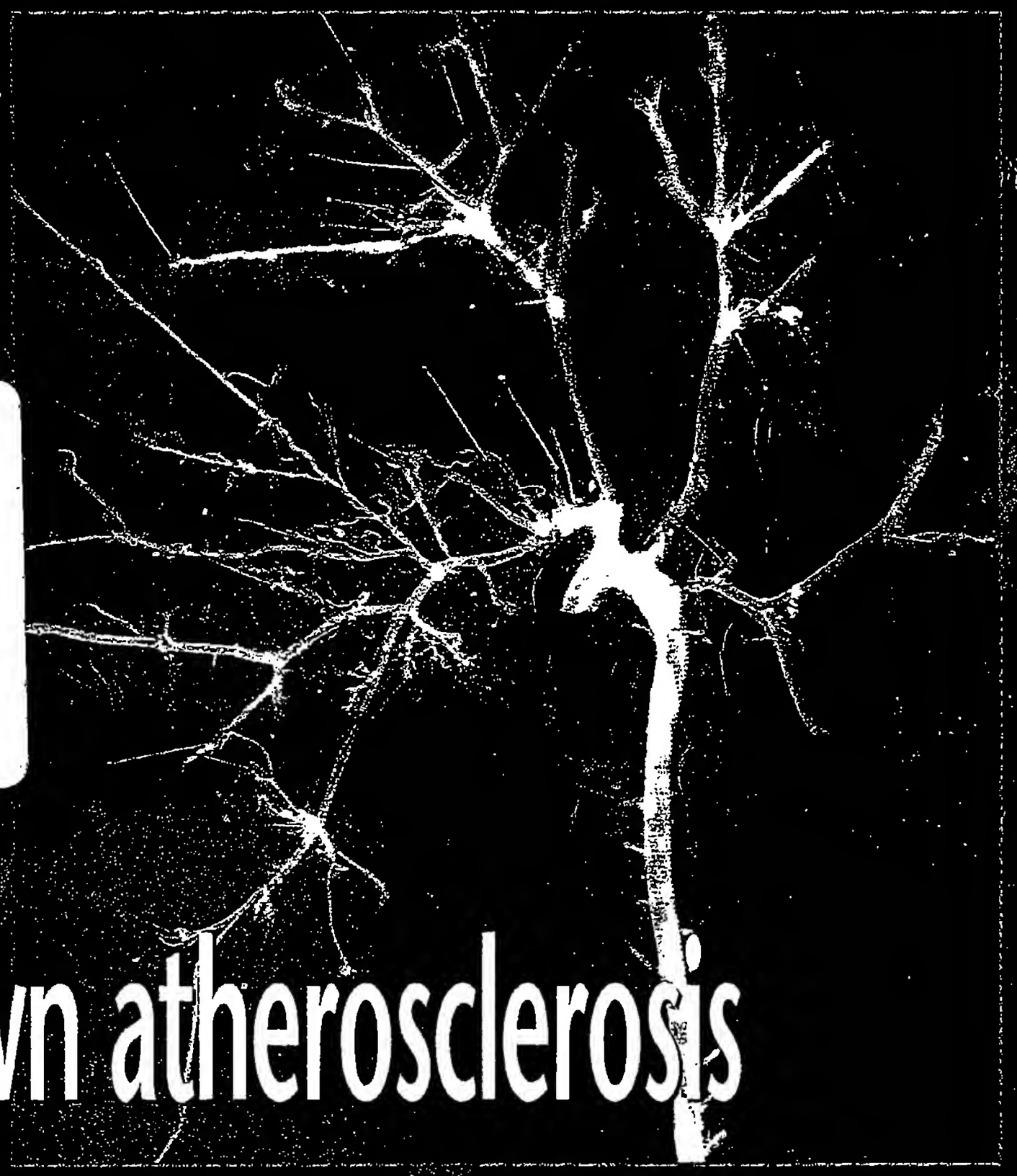
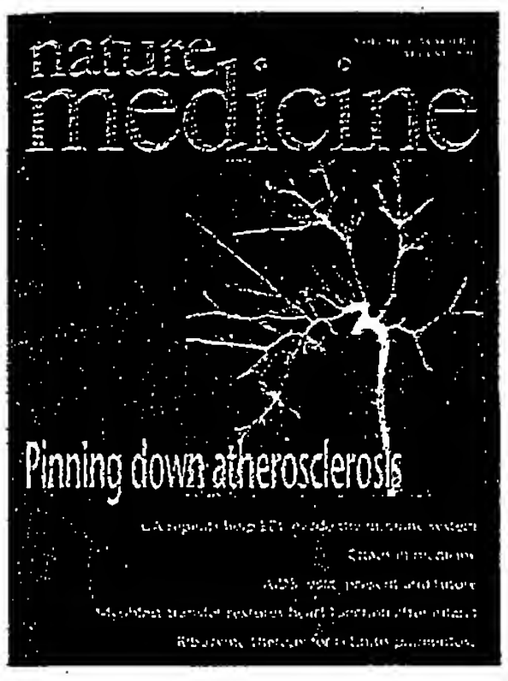
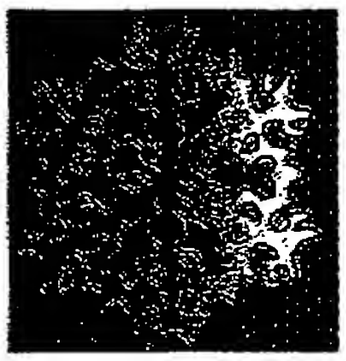


Pinning down atherosclerosis



Although several mouse models of human familial hypercholesterolemia have been described, due to intrinsic differences in mouse and human lipoprotein metabolism they do not accurately reflect the human condition. On page 934, Lyn Powell-Braxton and colleagues describe a new (LDL receptor- ApoB mRNA editing catalytic polypeptide-1-deficient) mouse that develops elevated LDL and extensive atherosclerosis similar to the human condition. The cover shows extensive atherosclerotic lesions present on the aorta of the mutant mouse.

Wellcome Trust says UK research still on the map. (page 871)



Chaos in medicine (page 882)

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EXHIBIT

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DNA Plasmid Based Vaccination Against the Oncogenic Human T Cell Leukemia Virus Type 1

M.G. AGADJANYAN[1,2], B. WANG[3], S.B. NYLAND[4], D.B. WEINER[1], and K.E. UGEN[4]

1 Introduction

The human T cell leukemia virus type 1 (HTLV-1) holds its place in history as the first human retrovirus established to be associated with disease. Since its discovery, it has been demonstrated to be the causal agent of adult T cell leukemia (ATL) and HTLV-1 associated myelopathy (HAM). In addition, more recently this retrovirus has been suggested to have a role in the etiology and/or pathogenesis of a myriad of autoimmune disorders including rheumatoid arthritis and uveitis (UGEN et al. 1996b). Also, the role of infection with HTLV in accelerating progression to acquired immunodeficiency syndrome (AIDS) in human immunodeficiency virus (HIV)-1 infected individuals has also been suggested (KRAMER et al. 1989). The worldwide seropositivity of HTLV-1 and the related HTLV-2 is estimated to be at

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least 10 million. Coupled with its association to fatal and debilitating disorders as well as its potential role in the pathogenesis of other serious illnesses an effective vaccine is warranted. In this review we will summarize current information on HTLV-1 including immune responses to this virus, correlates of protection as well as potential vaccination strategies including DNA plasmid inoculation.

2 General Biology of Human T Cell Leukemia Virus

The human T lymphotropic virus was first isolated by Robert Gallo's group (POIESZ et al. 1980, 1981). The newly discovered virus, which subsequently was named the human T cell leukemia virus, was the first human retrovirus for which a disease association was made (YOSHIDA et al. 1982). It was therefore designated the human T-cell lymphotropic/leukemic virus type 1, or HTLV-1. Shortly after the discovery of HTLV-1 another human T-cell leukemia virus, named HTLV-2, was isolated from another patient using a similar technique (KALYANARAMAN et al. 1982). An etiologic role for HTLV-1 in ATL and HAM has been demonstrated, but a disease association for HTLV-2 infection has not been established clearly, although some suggestion has been made that it may be associated with a neurodegenerative disorder similar to HAM (CANN et al. 1990; HALL et al. 1996). Both HTLV-1 and HTLV-2 are members of the oncovirinae subfamily of retroviruses. HTLV-1 and HTLV-2 have recently (COFFIN 1996) been placed in a separate group of which the bovine leukemia virus (BLV) is also a member (i.e., the HTLV/BLV subfamily). The discovery and description of HTLV-1 and -2 greatly facilitated the characterization of the etiological agents for AIDS, HIV-1 and -2, which belong to the subfamily of lentiviruses (BARRE-SINOUSSI et al. 1983; GALLO and JAY 1991). Therefore, there are two subfamilies of human retroviruses which infect human CD4-positive T cells but nonetheless produce different and opposite effects. While HIV-1 and 2 results in the death of $CD4^+$ T cells, HTLV-1 infection induces polyclonal and/or monoclonal T cell proliferation.

3 Molecular Biology of Human T Cell Leukemia Virus

The HTLV-1 proviral genome consists of 9032 nucleotides and includes regions coding for structural proteins (*gag* or *env*) and viral protease and polymerase (*pol*), similar to HIV-1. The genome also contains an additional sequence designated pX. This region is adjacent to the envelope (*env*) gene and contains three overlapping regulatory genes encoding the *trans*-activator protein (*tax* p40), transmodulator protein (*rex* p27), and a third gene which encodes for a protein (p21) whose function is unknown (NAGASHIMA et al. 1986; SAGATA et al. 1985; SEIKI et al. 1983). HTLV-1 is most closely related to HTLV-2, with a 66% sequence homology, but

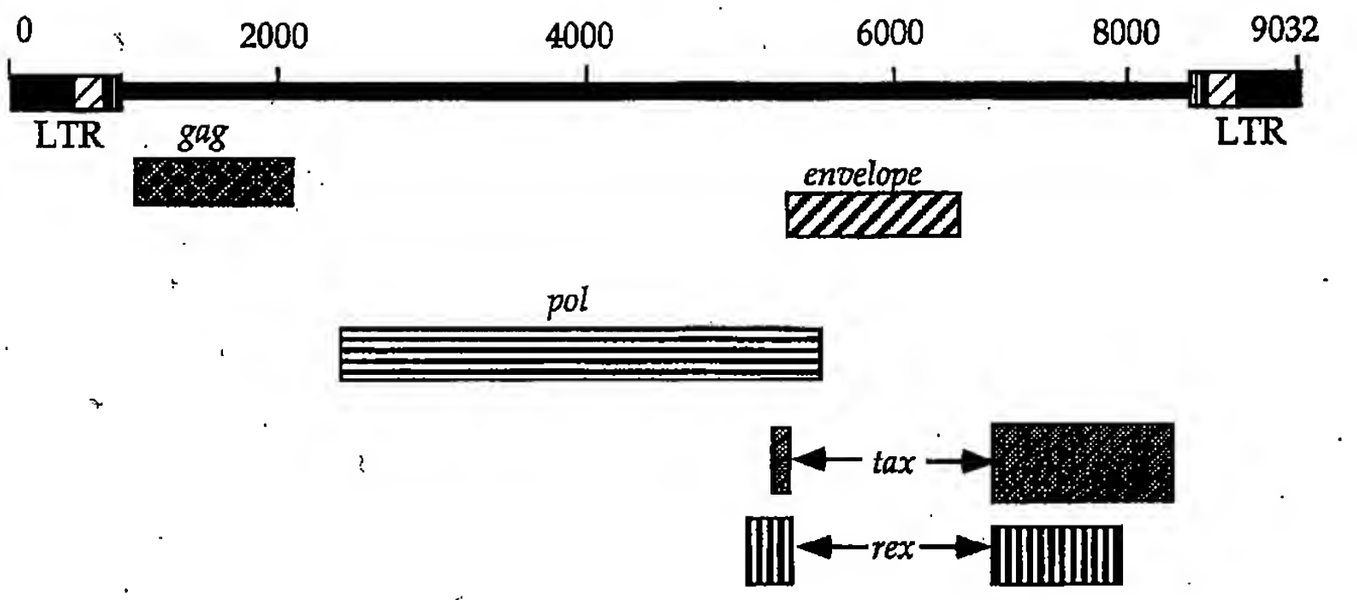

Fig. 1. Genome structure of the human T cell leukemia virus type 1 (HTLV-1). The topographical relationship of the important structural and regulatory genes of the human T cell leukemia virus genome is shown

shares high sequence homologies with additional retroviruses such as BLV and simian T cell leukemia virus. Figure 1 shows the relationship between the various genes of the HTLV-1 genome (YOSHIDA and SEIKI 1987). Unlike other oncoviruses, HTLV-1 does not express an analogue of any known cellular oncogene, nor does it insert its proviral genome into a specific regulatory region of the host DNA. The provirus acts randomly, taking up residence in any open region provided by the host cell's transcriptional activity. Although the mechanisms involved in the oncogenic transformation of infected cells are poorly understood, it is thought that the HTLV-1 *tax* may play an indirect role in transformation by its ability to associate with enhancer binding proteins of the host cell, such as CREB, CREM, NF-kB p50, and SRF (SUZUKI et al. 1993a, b; TANAKA et al. 1990). When *tax* interacts with these proteins, it enables the appropriate cell enhancer to bind, thus causing the up-regulation of transcription (SUZUKI et al. 1994). It has been hypothesized that this interaction involves activation of the interleukin (IL)-2 receptor α-chain, which would link the tax protein activity to the transformation event (INOUE et al. 1986). Another protein hypothesized to play a role in the process of cell transformation is the *rex* protein, which appears to regulate proliferation in a posttranscriptional manner (HIDAKA et al. 1988).

4 Pathogenesis of Human T Cell Leukemia Virus

The ability of HTLV-1 to up-regulate transcription of T cells or to induce their proliferation is linked to two disorders, i.e., ATL and HAM/TSP (tropical spastic pareparesis) (GESSAIN et al. 1985; SMITH and GREENE 1991). HAM/TSP is a neurodgenerative disorder which mimics some of the clinical manifestations of multiple

sclerosis (GESSAIN and GOUT 1992). HAM/TSP can develop within several months of infection by HTLV-1. In this disorder infected cells in the CNS present antigen, eventually leading to a chronic inflammatory state. The ensuing tissue damage is presented clinically as a progressive demyelinating syndrome with weakness starting at the extremities, and may resolve to varying degrees over time. In contrast to HAM/TSP, the second disorder, ATL, is attributed to the monoclonal proliferation of infected cells and presents abruptly during later adulthood with clinical latency lasting up to 50 years (KAPLAN and KHABBAZ 1993). ATL manifests clinically as a leukemic infiltration of a number of organ systems accompanied by moderate to profound immunosuppression (BUNN et al. 1983). The leukemia is frequently aggressive and generally untreatable, and death usually occurs within several months after diagnosis. Epidemiological studies of the two disorders have revealed factors suspected of influencing the direction of an HTLV-1 infection. These factors include the type and magnitude of the immune responses to HTLV-1 antigens, organ tropism, and the age at which an individual acquires an infection (RENJIFO et al. 1996). HAM/TSP is associated with infections acquired during early adulthood, while the emergence of ATL corresponds to a history of virus exposure from the prenatal period up through the first year of life, and breastfeeding appears to be an important epidemiological factor (HINO et al. 1996). In addition, certain geographical regions contain distinct pockets of high ATL and HAM/TSP incidence in conjunction with endemic HTLV-1 infection. These clusters are most prevalent in the Caribbean Basin (BLATTNER et al. 1982), Africa, and southwestern Japan (HINUMA et al. 1982; TAJIMA et al. 1982). Nagasaki has been shown to be especially vulnerable to HTLV-1 infection, with the prevalence of carriers being 10% in older persons and 4% in pregnant women (HINO et al. 1985). Overall, at least 10 million people are infected with HTLV-1/2 worldwide (DE THE and BOMFORD 1993). Individuals infected with HTLV-1 develop antibody responses against the virus and become lifelong carriers. It is estimated that one of every thousand infected individuals per year will become afflicted with ATL, with a lifetime risk of developing ATL being 2.5%–5%. The lengthy latency period has made the pathogenesis of HTLV-1 infection and its associated disease manifestations somewhat difficult to understand.

Transmission routes are better understood and have been established to be sexual spread, parenteral injection of contaminated blood, and mother-to-child (vertical) transmission. The evidence for male to female transmission of HTLV-1 is suggested by the prevalence of elderly female carriers (TAKATSUKI et al. 1982) who are wives of seropositive males and by the presence of HTLV-1 infected T cells in the infected husbands' seminal fluids (NAKANO et al. 1984). However, because this higher incidence of female carriers is noted only in female populations over 60 years of age, male to female sexual transmission of HTLV-1 is thought to be inefficient. Nearly 50% of patients receiving a blood transfusion from an HTLV-I infected source also become carriers of infection (HINO et al. 1985; OKOCHI et al. 1985). The most striking aspect of HTLV-1 transmission by blood transfusion is its reliance on cell to cell association, i.e., frozen cell-free plasma is not infectious. This property is consistent with tissue culture studies demonstrating that infection by HTLV-1 virions is much less efficient when cell-free viral suspensions are used rather than contact cell cultures

(AGADJANYAN et al. 1994a; CLAPHAM et al. 1983; DEROSSI et al. 1985; FAN et al. 1992; MIYOSHI et al. 1981; WEISS et al. 1985). Mother-to-child transmission of infection was first observed epidemiologically from the association of carrier clusters in families with the hallmark of a maternal seroconversion followed by HTLV-1 infection of children born after the conversion took place, while children born prior to the maternal seroconversion remained uninfected (TAJIMA et al. 1982). It was noted that in these clusters HTLV-1 infections occurred at a predominately early stage of life, which is compatible with the notion and importance of maternal-infant transmission. The genetic route for HTLV-1 infection was eliminated as a possibility when it was found that the virus is not endogenous to humans (YOSHIDA et al. 1982). Although a case is occasionally reported in which HTLV-1 infected cells are identified in the cord blood of infants born to infected mothers (KOMURO et al. 1983), this pre- and perinatal form of transmission does not appear to play a major role in the spread of the virus. Since the lag time for the development of antibodies is approximately 2 months from the time of infection by blood transfusion (OKOCHI et al. 1985) or by oral transmission (YAMANOUCHI et al. 1985), seroconversions from a perinatally acquired infection would be expected to take approximately 2–3 months to develop. Instead, seroconversions took at least 6 months to be detected and the majority of conversions did not occur until 12 months after birth (HINO et al. 1985) suggesting that vertical transmission of the virus could be attributed to a postnatal route, as in breast feeding. ATL prevention programs in Japan advise HTLV-1 infected mothers to avoid breastfeeding their infants. Although the program has helped to reduce the spread of HTLV-1 infection to some extent, it has proven impractical for other HTLV-1 endemic areas, such as the Caribbean basin countries where economic pressures preclude the use of expensive bottle-feeding equipment and formulas, and where the medical and educational resources of Japan are unavailable. More accessible forms of prevention, including an effective vaccine strategy, would better serve such communities. For this reason, the development of an HTLV-1 vaccine has become the focus of several research groups.

As indicated earlier it is estimated that at least 10 million individuals worldwide are infected with HTLV-1. This is certainly a sufficiently high enough incidence of infection to warrant the development of an effective vaccine. Coupled with the association of HTLV-1 infection with serious disorders as well as its potential role as a factor for the development of AIDS in HIV-1/HTLV-1 co-infected individuals an effective vaccine against HTLV-1 would be a significant development. In addition, strategies used to develop a vaccine against HTLV-1 may be applicable in the effort to develop an efficacious vaccine against HIV-1.

5 Vaccine Development

Currently accepted vaccine strategies generally employ two resources for producing immunogenic material – live infectious agents, or inactivated/subunit preparations.

The polio and smallpox vaccines are examples of the use of live, attenuated agents to stimulate protective T_{helper} (Th) and $T_{cytotoxic}$ (cytotoxic T lymphocyte, CTL) responses. This occurs during a nonpathogenic infection of the host and leads to an additional humoral immune response. The use of this type of vaccine for protection against human retroviruses still awaits the development of a nonpathogenic, yet immunogenic strain; an unlikely possibility for the near future. Subunit preparations include the recently developed hepatitis B surface antigen vaccine, which has been reported to stimulate protective T_{helper} and humoral immune responses. Although subunit vaccines appear to work with some pathogens, their effectiveness with other pathogens has not been confirmed. An additional strategy includes the vaccinia virus, which has been engineered to operate as an immunogenic expression vector and can elicit broader T cell immune responses (Moss 1988). It was initially believed that the vaccinia system would prove to offer a safe and simplified alternative to the other vaccination schemes currently available. However, it was later found that the viral vector itself presents antigens which can react with the immune system of the vaccinated individual, thus leading to a type of hypersensitivity reaction. This reaction renders the vaccinia construct impotent after a limited number of uses, as the construct would be neutralized before the desired immunogen could be processed.

The ideal vaccine for human retroviruses (i.e., HTLV-1 and HTLV-2) would act as an effective subunit or attenuated system, eliciting the whole spectrum of protective immune responses in the vaccinated subject without the risk of reversion to a pathogenic wild-type expression and without the risk of deleterious immune responses to the delivery construct. To this end, the DNA plasmid vaccination technology has been under investigation (Ulmer et al. 1993; Wang et al. 1993). This methodology involves the cloning of DNA sequences into an appropriate eukaryotic expression vector followed by delivery to the host through a number of different techniques. Genes cloned in this manner can be manipulated so that single proteins, or an extended genome excluding the genes that might lead to pathogenesis, can be presented.

The potential efficacy of the DNA plasmid vaccination system against human retrovirus (HIV-1) was also shown. We have been successful in eliciting neutralizing antibodies as well as CTL activity in rodents and nonhuman primates with HIV-1 envelope expressing constructs (Boyer et al. 1996; Ugen et al. 1996a, 1997; Wang et al. 1993, 1995). Importantly, this methodology has been successful in decreasing viral load in HIV-1 infected chimpanzees as well as protecting naive chimpanzees from heterologous challenge (Boyer et. al., 1997). This methodology can, in principle, be applied to other infectious agents such as HTLV-1.

5.1 Immune Response to HTLV-1

In order to design a vaccine for HTLV-1, it is necessary to document and understand the immune responses elicited against this retrovirus. Like its retroviral relatives, HIV-1 and -2, the envelope glycoproteins of HTLV-1 can act as major

antigens which are recognized by serum antibodies in infected individuals. The HTLV-1 transmembrane glycoprotein gp21 serves to anchor the exterior glycoprotein gp46 by a noncovalent association and assists in the attachment of gp46 to the host cell membrane (BOHNLEIN et al. 1991). Both gp46 and gp21 are derived from the proteolytic cleavage of an envelope precursor, gp66, which is encoded by the viral *env* gene (BOLOGNESI et al. 1978). Epitope mapping studies with the HTLV-1 envelope glycoprotein have not been as extensive as similar studies involving HIV-1, nonetheless several immunodominant B cell epitopes and a T cell epitope have been identified to date. A major neutralizing linear determinant has been identified in the external glycoprotein which spans amino acids 187–196. (DELAMARRE et al. 1994). Mutagenesis of this region has indicated its role in cell to cell fusion. Other regions within the external glycoprotein identified as of potential functional importance are amino acids 53–75 and 90–98 (DELAMARRE et al. 1994; PALKER et al. 1992) as well as two regions in the COOH-terminal amino acids 213–236 and 287–311 (BABA et al. 1993; LAIRMORE et al. 1992; LAL et al. 1991). One region in the gp21 transmembrane glycoprotein has been suggested to elicit neutralizing antibodies (i.e., amino acids 346–368) (DELAMARRE et al. 1994) in the central region of the molecule (amino acids 191–214) and another near the COOH-terminal (amino acids 242–257) (LAIRMORE et al. 1992; LAL et al. 1991). The external envelope glycoprotein gp46 also contains a region (amino acids 190–209) which was shown to be a target for cytotoxic T cell activity (PALKER et al. 1990).

HTLV-1 appears to be a good candidate for vaccine development because of the low variability within the envelope glycoprotein and the ability of this retrovirus to infect small animals; i.e., rats and rabbits (AMI et al. 1992; IBRAHIM et al. 1994; ISHIGURO et al. 1992; MIYOSHI et al. 1985). The hypervariability noted in HIV-1 can reduce the ability of neutralizing antibodies to appropriately interact with the immunogenic glycoprotein, thus reducing the efficacy of a vaccine designed against gp120 and this region in particular. With HIV-1, up to 27% of the envelope amino acid sequences may vary between different isolates (COFFIN 1986). Fortunately, this problem does not exist to any significant degree with HTLV-1. Envelope glycoprotein sequences from different HTLV-1 isolates were compared and demonstrated only a 2% overall variance in amino acid sequences, thus a high level of conservation was indicated for the HTLV-1 *env* sequence (GRAY et al. 1990). It is unlikely that a shift in variability will occur since mutations introduced into the HTLV-1 *env* gene results in a nonfunctional protein which is unable to form syncytia. When similar mutations are introduced into the HIV-1 genome, envelope function continues. This property of HTLV-1 suggests that it may contain the appropriate target material for the development of a broadly neutralizing vaccine based on the envelope glycoprotein.

In addition to the low variability in the envelope glycoprotein the important ability of anti-envelope antibodies in patient sera to cross-neutralize HTLV-1 strains originating from disparate geographic regions around the world suggests that a vaccine developed against one strain may be effective in neutralizing other strains (CLAPHAM et al. 1984; HOSHINO et al. 1985). Also, it appears that natural immunity to HTLV-1 can be effective since maternal antibodies are able to protect

newborns against congenital transmission including that which occurs by the epidemiologically important breast feeding route (TAKAHASHI et al. 1991).

5.2 Anti-HTLV Envelope Glycoprotein Responses by Conventional Vaccines

Other work on the demonstration of functional antibody responses involved the use of a vesicular stomatitis virus pseudotype displaying the HTLV-1 envelope. This preparation was used to demonstrate the neutralizing ability of antibodies specific for the envelope glycoproteins (CLAPHAM et al. 1984; HOSHINO et al. 1985). Experimental vaccination with these viral glycoproteins were also able to inhibit the fusion activity of whole HTLV-1 (HOSHINO et al. 1983; NAGY et al. 1983). Likewise, cynomologous monkeys were protected from HTLV-1 challenge when immunized with viral envelope components which were produced by genetically engineered bacteria (NACAMURA et al. 1987). In addition, viral envelope gene products are recognized by HTLV-1 seropositive patients (KANNER et al. 1986) and experimental animals treated with these reagents can produce neutralizing antibodies (KIYOKAWA et al. 1984; SAMUEL et al. 1984). Most neutralizing antibodies against retroviruses, including HIV-1 and HIV-2, are directed toward the envelope glycoprotein. In the case of HTLV-1, an attractive target for vaccine development can be found in this region of the virus.

Other strategies which have been utilized in HTLV-1 vaccine development include soluble HTLV-1 viral protein (DEZUTTI et al. 1990), synthetic peptides (TANAKA et al. 1994), recombinant vaccinia (FRANCHINI et al. 1995) and recombinant adenovirus (KAZANJI et.al., in press). To some degree, all of these preparations resulted in protection of experimental animal models (i.e., monkeys or rabbits) from infection by HTLV-1.

5.3 Anti-HTLV Envelope Glycoprotein Responses by DNA Plasmid Vaccines

5.3.1 Humoral Immune Responses

Using our studies on HIV-1 as a guide we have embarked on work which targets the HTLV-1 envelope glycoprotein for a DNA plasmid based vaccine. For DNA plasmid inoculation studies in rabbits we used a construct designated pcTSP/AT-K.env (REDDY et al. 1988) which contains the gene for gp46 + gp21 (i.e., external and transmembrane glycoproteins) from HTLV-1 (Fig. 2A). For rat studies we used a dual vector system, i.e., pgTAXLTR, which contains the genes for HTLV-1 *tax*, and *env* and pc*REX*,which contains the gene for HTLV-1 *rex* (RIMSKY et al. 1988) (Fig. 2B, C). The coding region for HTLV-1 *rex* and tax genes were included in the vector cocktail for the generation of immune responses against these important viral proteins. Therefore these constructs following experimental mutation

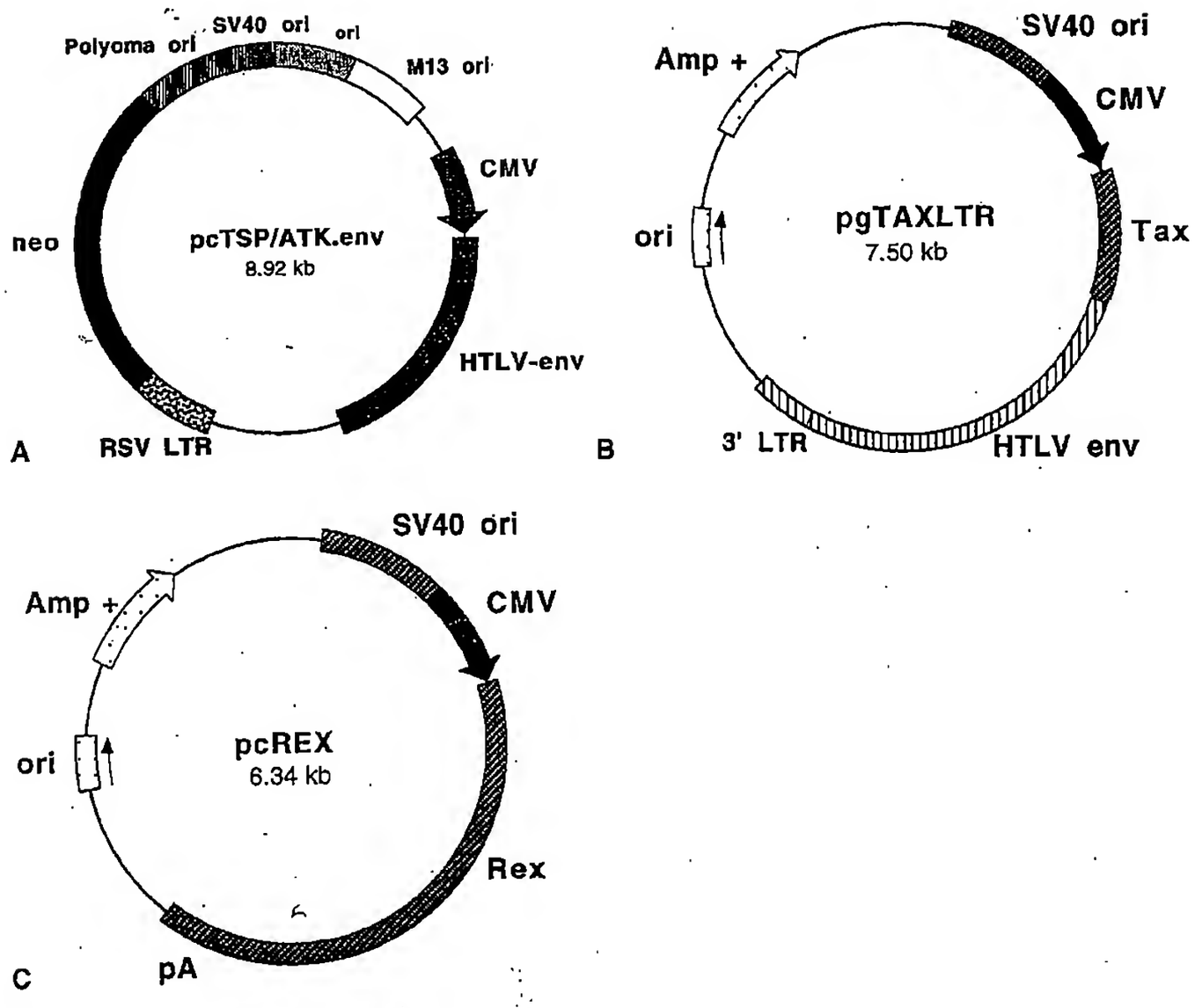

Fig. 2A–C. Human T cell leukemia virus type 1 (HTLV-1) envelope glycoprotein expressing DNA plasmids used in the study: A the pcTSP/ATK.env plasmid used in the rabbit inoculation experiments; B the dual pgTAXLTR and C pc *REX* plasmids used in the rat inoculation experiments

of the *rex* and tax genes could be in theory used as a therapeutic vaccine. Further analysis of immune responses against these viral antigens are planned. Possible up-regulation of the HTLV env protein synthesis by *rex* gene (HIDAKA et al. 1988) was another reason for including this gene into the vector.

Humoral immune responses were detected after single or multiple inoculations of 100 μg of the constructs after pretreatment with bupivacaine in both New Zealand White rabbits (AGADJANYAN et al. 1994b) as well as Fisher 344 rats (UGEN et al. 1995) (Table 1). Specifically immune responses in the experimental animals were evident, as measured by ELISA against several recombinant HTLV-1 envelope glycoproteins (i.e., RE-3: amino acids 165–306 and RE-6: amino acids 165–440) purchased from Repligen Corporation (Cambridge, MA). RE-3 contains the COOH-terminal half of gp46 while RE-6 contains the COOH-terminal half of gp46 + 75% of gp21. Both recombinant proteins contain the major neutralizing B cell epitopes of the glycoprotein as well as the T cell epitope. In addition, humoral immune responses were also measured against two peptides from gp46, designated Env-1 (amino acids 191-214) and Env-5 (amino acids 242–257) (RUDOLPH and LAL 1993). Also, immune responses in rabbits and rats to another described neutralizing epitope, amino acids 272–292, were noted in our study.

Table 1. Immune responses in experimental animals used as targets for HTLV-1 *env* DNA plasmid inoculation

DNA vaccine	Rabbit					Rat				
	1	2	3	4	5	1	2	3	4	5
HTLV pcTSP/ATK	+	+	+	+	+	nt	nt	nt	nt	nt
HTLV pcrex + pctax	nt	nt	nt	nt	nt	+	+	+	+	+

1, RE-3; 2, RE-6; 3, Env-1; 4, Env-5; 5, peptide 6; RE-3, amino acids 165–306; RE-6, amino acids 165–440; Env-1, amino acids 191–214 (LPHSNLDHILEPSIPWKSKLLTLV); Env-2, amino acids 242–257 (SPNVSVPSSSSTPLLY); peptide 6, amino acids 272–292 (NWTHCFDPQIQAIVSSPCHNS).

5.3.2 Neutralizing Antibody Responses

Anti-syncytial activity of antisera from rabbits and rats inoculated with an HTLV-1 envelope expressing constructs were measured by standard procedures (AGADJANYAN et al. 1994a; CLAPHAM et al. 1984). Rabbits were inoculated three times with 100 μg of an HTLV-1 envelope expressing construct (pcTSP/ATK.env) after pretreatment with bupivacaine (Fig. 3A). Fifteen weeks after the final inoculation animals were bled and the ability of the antisera to inhibit syncytia between HTLV-1 infected (MT-2) cells and a target B cell line (BJAB-WH) was tested. Sera were mixed with both cell lines and 48 h later syncytia formation was quantitated. Rabbit 1 was inoculated with a control plasmid which did not express the HTLV-1 envelope glycoprotein. Significant inhibition of syncytia was noted in all three of the rabbits which were inoculated with the HTLV-1 envelope expressing plasmid while the control rabbit failed to inhibit syncytia formation (AGADJANYAN et al. 1994b). Likewise, Fisher 344 rats were inoculated with either (a) 100 μg of an HTLV-1 envelope expressing constructs (i.e., pgTAXLTR + pc*REX*) after pretreatment with bupivacaine, (b) 5 μg of a recombinant HTLV-1 protein (RE-6) or (c) a control DNA plasmid not expressing the HTLV-1 envelope construct (Fig. 3B). Twelve days later the rats were bled, sera collected and the ability to inhibit syncytia formation was performed using the cell lines and conditions described above. Inhibition data is shown for pre- and post-inoculation bleeds. Rats inoculated with either the experimental DNA plasmid or the recombinant protein demonstrated an ability to inhibit syncytia formation. In fact, DNA plasmid inoculation appeared to produce a more potent neutralization as evidenced by the higher percent inhibition of syncytia at a serum dilution of 1:27 when compared to serum from rats vaccinated with recombinant protein (UGEN et al. 1995).

5.3.3 Cellular Immune Responses

T cell proliferative responses in rabbits inoculated with an HTLV-1 envelope expressing DNA plasmid were measured. The rabbits were inoculated intramuscularly with 100 μg of the pcTSP/ATK.env plasmid 24 h after pretreatment with

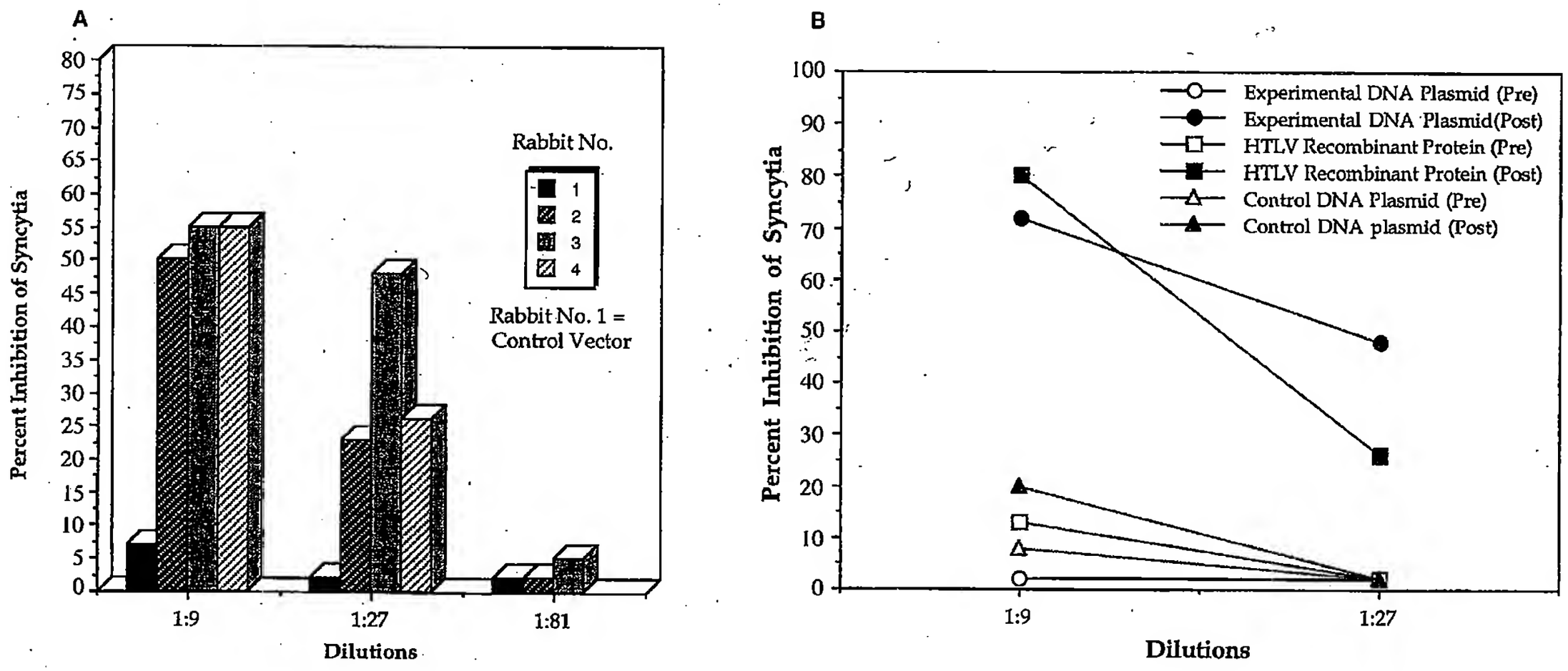

Fig. 3A, B. Anti-syncytial activity of antisera from rabbits and rats inoculated with an HTLV-1 envelope expressing construct. **A** Rabbits 1–3 were inoculated with the pcTSP/ATK.env plasmid while rabbit 4 received a control plasmid with the specific HTLV-1 gene sequence deleted. **B** Rats were inoculated with the pgTAXLTR and pc*REX* plasmids, an HTLV-1 recombinant envelope glycoprotein plasmid (RE-6) or a control plasmid which had the HTLV-1 envelope gene deleted. *Pre* refers to a pre-inoculation measurement and *post* refers to a post-inoculation measurement. Details of the methods are included in the text

0.5% bupivacaine. Blood was subsequently collected from the rabbits after vaccination and peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with either a positive control (PHA, phytohemagglutinin, at 4 μg/ml) or a specific HTLV-1 envelope recombinant protein (RE-6) at 1 μg/ml. Rabbit 1 was inoculated with a control plasmid which did not express the HTLV-1 envelope glycoprotein. Specific proliferation was measured by incorporation of [^{3}H]thymidine (AGADJANYAN et al. 1994b). The results indicate that specific proliferation occurred in the rabbits inoculated with the HTLV-1 *env* expressing plasmid (Fig. 4).

5.3.4 Protection from HTLV-1 Infection by DNA Plasmid Based Vaccine

The ultimate test of the efficacy of a vaccine is its ability to prevent infection and/or disease. To this end Fisher rats which were inoculated with an HTLV expressing construct and which demonstrated significant anti-envelope glycoprotein antibodies were challenged with HTLV-1-infected MT-2 cells. The protocol for this viral challenge is as follows:

Fisher 344 rats were vaccinated three times (following pre-treatment with 0.5% bupivacaine-HCl) with 100 μg of the HTLV-1 DNA plasmids (pgTAXLTR +

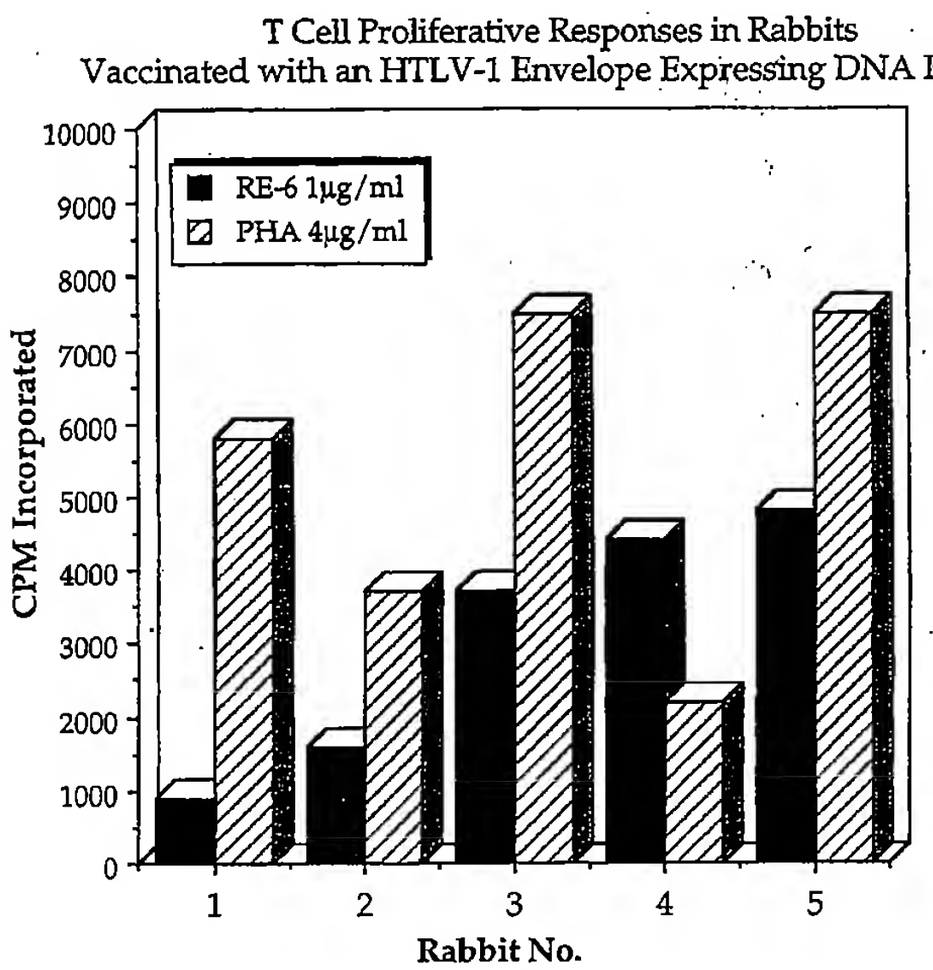

Fig. 4. T cell proliferative responses in rabbits inoculated with an HTLV-1 envelope expressing DNA plasmid. Rabbit 1 was inoculated with a control DNA plasmid in which the HTLV-1 envelope gene was deleted. Rabbits 2–5 were inoculated with the HTLV-1 envelope expressing the pcTSP/ATK.env plasmid. Details of the assay are included in the text

pc*REX*) at 2 week intervals. In addition other rats were inoculated with a control plasmid according to the same schedule. Humoral immune responses against an HTLV-1 envelope glycoprotein were measured in the rats inoculated with the HTLV *env* plasmid. Two weeks following the final inoculation all of the rats were challenged with 3×10^6 HTLV-1 infected human MT-2 cells by intraperitoneal injection. Twelve weeks after challenge with MT2 cells the rats were bled and DNA was prepared from the PBMCs and subjected to PCR using the following primers from the pX gene region (no. 1 = nt 7336–7355: CGGATACCCAGTCTACG-TGT and no. 2 = nt 7473–7492: CT*GAG*CCGATAACGCGTCCA). Figure 5 shows the results of these analyses. The gel lane designations for the figure are as follows: lane 1, DNA size markers; lane 2, DNA from HTLV-1 infected MT-2 cells; lane 3, DNA from one of the MT-2 challenged rats vaccinated with the HTLV-1 env DNA plasmid in which actin specific primers were used in the reaction as a control; lanes 4 and 5, DNA from rats inoculated with control plasmid; lanes 6 and 7, DNA from rats inoculated with experimental HTLV-1 *env* expressing plasmid. These results demonstrated that the vaccinated rats had the px PCR product absent from the DNA of their PBMCs. The positive actin band (lane 3) suggested that DNA was present in these samples and the lack of px signal (lanes 6, 7) was not due to an absence or low concentration of DNA.

Recently KAZANJI et al. (in press) have also reported partial protection from infection by cell associated HTLV-1 in rats vaccinated with an HTLV-1 env expressing construct. In this study they found anti-envelope CTL activity, but not humoral immune responses against HTLV-1 env. Based upon these findings they concluded that CTL activity elicited by the DNA plasmid inoculation technique was correlated with protection. In our study CTL activity was not measured, however, humoral neutralizing antibodies were present in the rats, which were

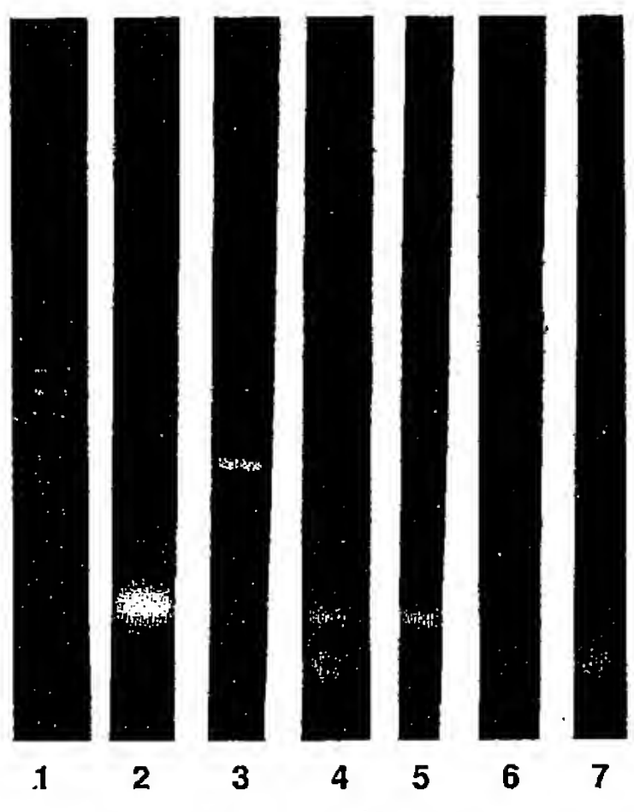

Fig. 5. Protection of Fisher 344 rats from infection with HTLV-1 MT-2 cells by HTLV-1 envelope glycoprotein DNA plasmid inoculation. Details and a description of the analysis are included in the text

protected after challenge. It is clear than additional studies are necessary to establish the relative protective roles of cellular and humoral immunity against HTLV-1 infection and disease.

6 Discussion and Summary

Successful vaccine development for human retroviral infections are of considerable importance due to the worldwide AIDS pandemic. However, the first human retrovirus described which produces disease, HTLV-1, also infects at least 10 million people worldwide and results in fatal and debilitating disorders. Therefore, an effective vaccine for this retrovirus is also warranted. Several characteristics of HTLV-1 suggest that a vaccine based on the envelope glycoprotein may be feasible. These include: (a) minimal hypervariability of the envelope glycoprotein (unlike HIV-1), indicating that a vaccine may be effective against all viral isolates and (b) the ability of envelope glycoprotein vaccinogens to protect monkeys from challenge with cell associated HTLV-1. Also, some evidence suggests that humoral immune responses alone may be sufficient to protect against infection. Cellular immune responses, however, may also play some role in protection. Therefore, an HTLV-1 envelope glycoprotein expressing DNA plasmid vaccine may be useful in eliciting protective immune responses against this retrovirus. This review has summarized the current knowledge concerning HTLV-1 biology and pathogenesis as well as the immune responses elicited against this retrovirus during natural infection and experimental vaccination. We have summarized our work to date on the HTLV-1 envelope DNA plasmid vaccines in the rabbit and rat infection models for this retrovirus. Humoral and cellular immune responses in the animal systems have been demonstrated. Importantly, current studies indicate that this vaccination methodology can protect rats from infection with HTLV-1. Recently, other authors have also reported protection of rats from infection by HTLV-1 by vaccination with envelope expressing DNA constructs. All the data reported here suggest the potential utility of the DNA plasmid vaccine technique against HTLV.

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Current Topics in Microbiology and Immunology

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Cover Illustration: Plasmid delivery to a host tissue results in tissue specific protein production and specific activation of T cells, B cells and antigen presenting cells generating specific immunity (by courtesy of Bin Wang and Michael Chattergoon).

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Review Article

Therapeutic Vaccine for Lymphoma

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The unique antigenic determinants (Idiotype [Id]) of the immunoglobulin expressed on a given B-cell malignancy can serve as a tumor-specific antigen for active immunotherapy. Therapeutic vaccines targeting the tumor-specific idiotype have demonstrated promising results against lymphomas in phase I/II studies and are currently being evaluated in phase III randomized trials. Additional vaccine therapies being developed include those based on DNA, dendritic cells, gene-modified tumor cells. It is hoped that immunotherapeutic agents, used in tandem or in combination, may in the future allow effective treatment of lymphoid malignancies and delay or even replace the need for conventional cytotoxic therapies.

Key Words: Idiotype, immunotherapy, lymphoma, vaccine

INTRODUCTION

Immunotherapy has now become an important part of our therapeutic armamentarium for hematologic malignancies. Several monoclonal antibodies have been approved by the FDA and are in widespread use either alone or in combination with chemotherapy or with other biologic agents. Other passive therapies with various immune cell populations are under investigation. Active immunotherapy, whereby the host is induced to make an immune response against its own tumor cells, has long been a goal of tumor immunologists.

The central hypothesis of active immunotherapy of cancer is that either the tumor cell itself or antigens derived from the tumor cell (which are specific, or at least selective, for the tumor cell) can be modified and injected back into the patient as a therapeutic (not preventive) vaccine. The desired result is activation of both major arms of the immune response, the host antibody response and potentially a host T-cell response, against the target tumor cell or antigen, thereby aiding in the eradication of the disease.

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Idiotype as a tumor-specific antigen for B-cell Non-Hodgkin's lymphoma

Many of the efforts toward the development of a vaccine against human malignancies have been frustrated by the lack of identification of a tumor-specific antigen that would allow tumor cells to be distinguished from normal cells. In the case of B-cell NHL however, the tumor-specific immunoglobulin expressed on the surface of malignant B-cells can function as a tumor-specific antigen and has been exploited as a target for active immunotherapy. Each B lymphocyte expresses an immunoglobulin molecule on its surface, which is capable of recognizing and binding to a unique antigen. The variable region of the immunoglobulin that binds to the antigen is the product of a unique combination of gene sequences, and is referred to as its idiotype (Id) (Fig. 1). Since B-cell NHLs are composed of clonal proliferation of mature resting and reactive lymphocytes, which express synthesized immunoglobulins on the cell surface, the idiotypic determinants of the surface immunoglobulin of a B-cell lymphoma can serve as a tumor-specific marker for the malignant clone.

Although not entirely applicable to Id, the major limitation to using self-tumor antigens for cancer vaccine is that they are protected by self-

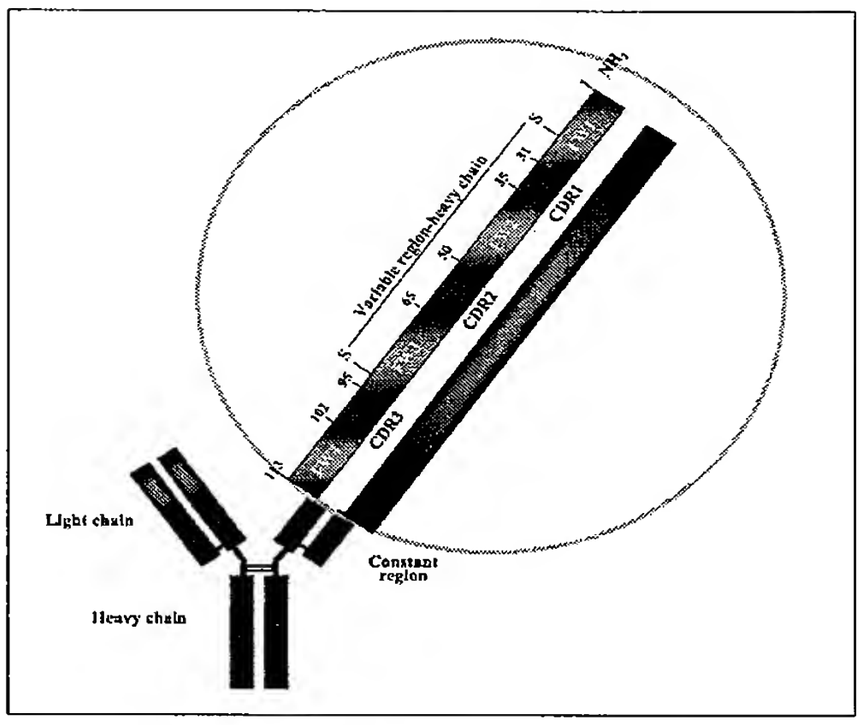

Fig. 1. Idiotype as a tumor antigen specific for B cell lymphoma. Malignancies of mature and resting B cells arise from clonal proliferation of cells that express immunoglobulins on their cell surface. Immunoglobulin molecules are composed of heavy and light chains, which possess highly specific variable regions at their amino termini and constant regions at their carboxy termini. The variable regions contain unique determinants termed idiotype (Id) that can be recognized as an antigen. The unique antigenic determinants are most likely derived from the hypervariable complementarity-determining regions (CDR), but not framework (FW) regions of the variable regions of heavy and light chains.[18] Amino acid numbers in the variable heavy chain region are shown from the amino (NH_2) terminus end.

tolerance mechanisms. The first step in cancer vaccine development is therefore to answer the question of whether it is possible to render an inherently weak tumor antigen immunogenic. This is largely a scientific question, one that should be answered in the affirmative before asking the medical question of whether cancer vaccines can induce meaningful clinical benefit. In the case of lymphoma Id vaccine development, it has taken 10 years to answer this scientific question in a definitive manner.

Preclinical studies of Id vaccine

In the early 1970s, Lynch and Eisen demonstrated in mice that active immunization with purified immunoglobulins (Id) from mineral-oil-induced plasmacytomas (MOPCs) induced Id-specific tumor resistance.[1,2] This phenomenon has been reproduced subsequently in a number of lymphoma, myeloma, and leukemia models.[3-11] In 1987, Kaminski et al., demonstrated that optimal immunization required conjugation to a strongly immunogenic carrier protein, such as KLH.[12] Subsequently, Kwak and colleagues demonstrated that the use of GM-CSF as an adjuvant facilitated the induction of tumor-specific $CD8^+$ T cells and enhanced the efficacy of the vaccine in a murine 38C13 lymphoma model.[13] GM-CSF likely acts by recruiting and promoting maturation of professional antigen-presenting cells such as dendritic cells, which may in turn activate pathways of antigen processing that allow exogenous proteins to be presented by class I molecules.[14] Animal studies also demonstrated that idiotypic vaccination conferred protection against tumor challenge and could cause regression of established tumor. Taken together, these results provided the rationale for testing autologous tumor-derived idiotypic surface immunoglobulin as a therapeutic "vaccine" against human B-cell NHL.

Phase I clinical trial of Id-KLH vaccine

The first human study of Id vaccination was pioneered by Kwak et al., in patients with follicular lymphoma (Table 1), which was a pilot study designed to determine whether it was possible to immunize against the Id portion of the protein.[15] Follicular lymphoma patients in minimal residual disease or complete remission after chemotherapy, were immunized with subcutaneous injections of autologous purified tumor-derived immunoglobulin, conjugated to KLH. Because no Id-specific immune responses were observed before the addition of an immunologic adjuvant to the first group of patients, patients subsequently (nine patients) received the entire series of immunizations with a standard emulsion adjuvant (Syntex adjuvant formulation 1 - SAF-1). In all, 41 patients were treated on this pilot study; and 41% demonstrated specific anti-Id antibody and 17% demonstrated cellular proliferative responses.[15] Of the 20 patients with residual disease following chemotherapy, two patients had complete regression of the tumor in association with the development of a specific immune response. Thus, these results were important because they demonstrated that patients with lymphoma could be induced to make sustained

Id-specific immune responses by active immunization with purified autologous tumor-derived surface Ig conjugated to the immunogenic carrier KLH. Furthermore, the induction of Id-specific immune responses was demonstrated in the setting of minimal tumor burden after conventional chemotherapy.

Phase II clinical trial of Id-KLH vaccine

Based on the preclinical observation that the addition of GM-CSF as an adjuvant to the vaccine induced tumor-specific $CD8^+$ T cells,[13] Bendandi and colleagues conducted a Phase II clinical trial where 20 previously untreated follicular lymphoma patients were treated with autologous tumor-derived Id-KLH+GM-CSF vaccine following induction of clinical remission with chemotherapy[16] (Table 1). This produced a homogeneous group of patients, all in first complete remission (CR), who were given vaccine treatment in the setting of minimal residual disease. The vaccine was injected subcutaneously in 5 monthly doses starting approximately 6 months after completing chemotherapy to allow time for immunological recovery. The vaccine was well tolerated with the main adverse effects being injection site reactions such as erythema, induration, and pruritus. There were no long-term adverse effects due to the vaccine.

Following vaccination, anti-KLH antibody and cellular responses were induced in all patients. Anti-idiotype antibody responses were induced in 15 out of 20 (75%) patients and Id-specific and/or tumor-specific $CD4^+$ and $CD8^+$ T-cell responses were observed in 19 out of 20 (95%) patients.[16] Importantly, significant levels of HLA class I-restricted killing of autologous tumor targets were also demonstrated in the vast majority of patients, suggesting the induction of a cytotoxic $CD8^+$ T-cell response. The specificity for this response was shown by the lack of killing of nonneoplastic, normal B cells from the same patients.[16] Further characterization of anti-idiotype cellular immune responses demonstrated that the T cells specifi-

Table 1. Published Clinical Trials of Idiotype Vaccination in Lymphoma

Formulation	No. of patients	Histology	Anti-Id/tumor immune response (%)		Comments	Ref
			Ab	T cell		
Id-KLH+SAF	41	FL	41	17	First human trial of Id vaccine	15
Id-KLH+GM-CSF	20	FL	75	95	Molecular remissions in 8/11 patients	16
Id-DC/Id-KLH-DC	35	FL	26	49	Clinical responses in 22% of patients	33, 34
Plasmid DNA	12	FL	0	8	Poorly immunogenic	56
Id-KLH+SAF	9	FL	89	N/A	Molecular remissions in 3/5 patients	57
Liposomal Id/IL-2	10	FL	40	100	Sustained T cell responses beyond 18 months	23
Id-KLH+GM-CSF	26	MCL	30	87	T cell responses induced in the absence of B cells	39
Id-KLH+GM-CSF	25	FL	52	72	Specific immune response associated with improved DFS	58
Fab+MF59+GM-CFS	18	FL, MM, DLBL, CLL, MCL, LPL	29	47	Specific immune response despite profound immunosuppression	59
Id-KLH+GM-CSF	31	SLL, FL	20	67	Clinical responses in 12.9% of patients	60

Id, idiotype; KLH, keyhole limpet hemocyanin; SAF, syntex adjuvant formulation; GM-CSF, Granulocyte-Macrophage Colony Stimulating Factor; DC, dendritic cell; FL, follicular lymphoma; MCL, mantle cell lymphoma; DFS, Disease-free survival; MM, multiple myeloma; DLBL, diffuse large B cell lymphoma; CLL, chronic lymphocytic leukemia; LPL, lymphoplasmacytic lymphoma; SLL, small lymphocytic lymphoma; N/A, not assessable.

cally recognized multiple unique immunodominant epitopes within the hypervariable complementarity-determining regions (CDR), but not framework regions of immunoglobulin heavy chain.[17] Monitoring of the patients for minimal residual disease showed that 8 out of 11 patients with PCR-positive t(14;18) chromosomal translocation breakpoints converted to PCR negativity in their blood immediately after completing vaccination and sustained their molecular remissions for a median of 18+ months (range: 8+ to 32+ months).[17] Thus, these results provided the first convincing evidence of an antitumor effect of Id vaccination. Analysis of time to relapse also provided an independent indication of clinical benefit. With a median follow-up of 9.2 years, median disease-free survival (DFS) is 8 years, and the overall survival rate is 95%.[18] While definitive statements cannot be made, because this was not a randomized trial, the DFS appears superior to that of a historical, ProMACE chemotherapy-treated control group (median DFS, about 2.2 years).[19]

In conclusion, this Phase II clinical trial demonstrated that tumor-specific $CD8^+$ T cells, capable of killing autologous tumor cells, could be induced by Id-KLH vaccination in combination with GM-CSF.[17] This study also established GM-CSF as an essential component of this vaccine, as an earlier study using the same immunogen, administered without GM-CSF, showed humoral but no $CD8^+$ T-cell responses.[15]

Phase III clinical trials of Id-KLH vaccine

The encouraging immunological and clinical outcome of the Id-KLH + GM-CSF vaccine in the Phase II clinical trial in patients with follicular lymphoma led to the initiation of a randomized double blind placebo controlled multicenter Phase III clinical trial to definitively answer the question of clinical benefit induced by idiotype vaccination. This Phase III trial was initiated by the National Cancer Institute, National Institutes of Health, Bethesda, MD and now sponsored by Biovest International, Inc. This trial was designed similar to the Phase II trial[16] where previously untreated advanced stage follicular lymphoma patients initially underwent an excisional lymph node biopsy and were treated into clinical remission with a PACE chemotherapy regimen. Patients who achieve a CR or CRu (complete response unconfirmed) are randomized in a 2 : 1 manner either to the specific vaccination arm of Id-KLH+GM-CSF or the non-specific vaccination arm of KLH+GM-CSF. The primary endpoint for this trial is to compare the disease-free survival between the two arms.[20] The secondary objectives of this trial are 1) to determine the ability of Id vaccine to produce a molecular CR in patients in clinical CR but PCR evidence of minimal residual disease after standard chemotherapy; 2) to evaluate the impact of Id vaccine to generate humoral and cellular immunologic responses against autologous tumor; and 3) to compare the overall survival of patients randomized to receive either the autologous tumor-derived Id vaccination (Id-KLH + GM-CSF) or non-specific vaccination (KLH + GM-CSF).

The Phase III trial was amended in July, 2006 to include administration of CHOP-R chemotherapy for remission induction prior to vaccine administration. The rationale for this was based on the fact that rituximab-based combination chemotherapy regimens were recently shown to induce a survival benefit in patients with follicular lymphoma and due to the fact that the induction of anti-tumor T cell immunity by idiotype vaccine was not impaired by the administration of prior rituximab containing chemotherapy. In the amended version of the trial, 540 patients will be randomized in a 2 : 1 ratio of Id-KLH vaccine + GM-CSF to KLH-KLH control vaccine + GM-CSF. The calculated trial size is sufficient to allow approximately 80% power to detect a difference between disease-free survival curves with an initial hazard ratio of 1.0 for the first 8 months and then an intended hazard ratio of 2.0 thereafter. The entire population of patients, whether induced with PACE or CHOP-R, will be analyzed in a combined manner. Patients will be stratified according to the International Prognostic Index, number of chemotherapy cycles, and type of chemotherapy.

Two additional randomized Phase III trials are currently evaluating the clinical efficacy of Id-KLH vaccine. These trials differ primarily in terms of the induction therapy and the method of idiotype production. The Genitope-sponsored trial

uses cyclophosphamide, vincristine, and prednisone (CVP) chemotherapy,[21] and the Favrille-sponsored trial uses the single agent anti-CD20 monoclonal antibody rituximab.[22] Moreover, while only CR and CRu patients are vaccinated on the Biovest study, both CR, CRu, and PR patients are vaccinated on the Genitope trial, and CR, CRu, PR and stable disease patients are vaccinated on the Favrille trial. As opposed to the hybridoma method in the Biovest study, the Genitope and Favrille trials use recombinant DNA technology for production of idiotype protein, but with different immunoglobulin backbone structure and the cellular systems used for Id protein expression. Of these, Id protein of Genitope trial contains a human IgG3 heavy chain backbone is produced by plasmid transfection of murine lymphoma cells, whereas that of Favrille trial has IgG1 backbone and produced in insect cells. The results of interim analysis from these trials are expected within the next two years and it would be interesting to see whether the idiotype vaccines can induce clinically meaningful benefit in patients with both minimal residual disease (Biovest study) as well as with low tumor burden (Genitope and Favrille studies).

Second generation vaccines

Given that the general question of whether it is possible to immunize against Id has been answered by the completed phase II trial, and that a randomized controlled phase III clinical trial has been opened to answer the question of clinical efficacy, a third major research objective is to streamline the production of these individualized vaccines to make this therapy more practical. Accordingly, alternative methods for formulating Id, an otherwise nonimmunogenic antigen, into an immunogenic vaccine are being tested in preclinical syngeneic murine lymphoma models.

Liposomal idiotype vaccines

A strategy of replacing KLH with a more uniform liposomal carrier, containing dimyxistoyl phosphatidyl choline (DMPC) lipid and recombinant human interleukin (IL)-2 was explored and revealed that this liposomal carrier reproducibly converted lymphoma Id into a tumor rejection antigen.[23] Head to head comparisons against Id-KLH (as well as Id- KLH + GM-CSF), controlled for Id antigen dose, revealed equivalent to superior potency for the liposomal vaccine. On the basis of these results, a pilot clinical trial of this formulation was performed and the results demonstrated that liposomal delivery of Id was well tolerated and induced sustained tumor-specific $CD4^+$ and CD8+ T-cell responses in lymphoma patients.[24]

DNA vaccines

One of the major drawbacks of Id protein vaccines is that the vaccine production is time consuming and laborious. The idiotype for these protein vaccines is generated by hybridoma tissue culture technology (Fig. 2). An alternative to idiotype protein vaccination is to use DNA vaccines. Any delivery system that does not require protein expression holds tremendous potential for the goal of streamlining vaccine production. Immunoglobulin variable genes specific for the B-cell malignancies can be readily cloned[25,26] and combined into single chain variable fragment (scFv) format, encoding a single polypeptide consisting solely of VH and VL genes linked together inframe by a short, 15 amino acid linker. Preliminary studies in mice and humans showed that the DNA vaccine is weakly immunogenic in most cases and needs to

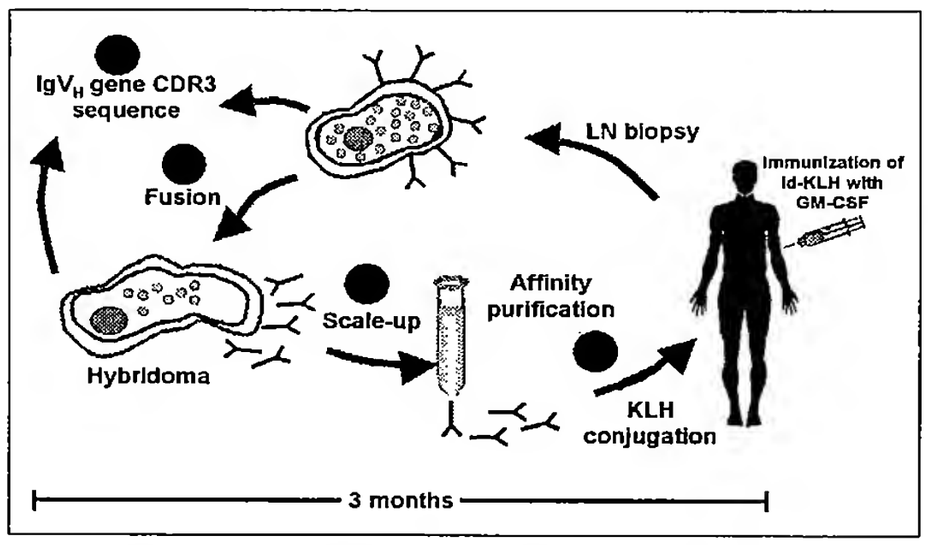

Fig. 2. Schematic diagram showing the production of Id protein vaccine using hybridoma technology. Id vaccines are custom-made from each patient's own tumor cells by fusion to the immortal myeloma cells. The Id protein is then chemically linked to the foreign protein KLH, combined with an immune-adjuvant and injected under the skin. IgV_H, immunoglobulin heavy chain variable region; CDR3, complementarity-determining region 3.

be used together with an adjuvant to render it immunogenic. King et al. demonstrated that fusion of the gene encoding fragment C of tetanus toxin to scFv markedly enhances the anti-idiotypic antibody response and induced protection against B-cell lymphoma in mice.[27,28] Biragyn and colleagues showed that the efficiency of DNA vaccination *in vivo* could be greatly increased by encoding a fusion protein consisting of idiotype (scFv) fused to a proinflammatory chemokine moiety that facilitates targeting of antigen-presenting cells for chemokine receptor-mediated binding, uptake, and processing of scFv antigen for subsequent presentation to $CD4^+$ or $CD8^+$ T cells, or both.[29-31] Specifically, mice immunized by gene gun with plasmids encoding monocyte chemotactic protein 3 (MCP-3) or interferon inducible protein 10 (IP-10)-scFv fusions, but not scFv alone, induced protective antitumor immunity against a large tumor challenge (20 times the minimum lethal dose). Furthermore, the level of protection was equivalent or superior to that of the prototype Id-KLH protein vaccine.

Cell based therapeutic vaccine approach

Dendritic cell vaccines

In the past several years, DCs have been identified as the most powerful professional APCs. Dendritic cells can take up, process, and present antigen in the context of co-stimulatory signals required for activation of both $CD4^+$ and $CD8^+$ T cells. In recent years, several strategies have been developed to exploit the antigen-presenting properties of DCs. Timmerman et al demonstrated in a murine lymphoma model that vaccination with Id-KLH-pulsed DCs induced superior tumor-protective immunity than did native Id-pulsed DCs.[32] In a pilot study, Hsu and colleagues used autologous DCs pulsed *ex vivo* with tumor-specific idiotype protein as a vaccine in 4 follicular B-cell lymphoma patients.[33] Subsequent clinical trial on 35 patients with follicular lymphoma treated with the same strategy showed a 22% overall clinical response.[34] This study demonstrated the feasibility and safety of Id-pulsed DCs as a vaccination strategy in humans.

Tumor cell vaccines

Immunization with irradiated, GM-CSF-transduced tumor cells can elicit cell mediated immunity against tumor antigens released by dying tumor cells and thereby resist growth of non-transfected tumor cells. Once again, GM-CSF serves to activate local antigen-presenting cells to efficiently take up and present these antigens to T-cells. In phase I/II studies of this approach in melanoma, renal cell carcinoma, and lung cancer, occasional clinical responses have been seen.[35-37] Levitsky et al showed that immunization of mice with lymphoma cells genetically engineered to produce GM-CSF, and to a lesser extent cells producing IL-4, eradicated pre-established systemic lymphoma.[38] The therapeutic effect of the GM-CSF- or IL-4-transfected lymphoma cells required both $CD4^+$ and $CD8^+$ T cells. In addition, the T-cell responses were shown to be Id specific in these mice, suggesting that GM-CSF-transduced tumor cell-based vaccination can induce immune responses against a native tumor antigen.

Conclusions and future prospects

Idiotype vaccination appears to be safe and immunogenic in patients with non-Hodgkin's lymphoma. The immune response appears to be directed against the tumor but not autologous normal B cells suggesting that idiotype is a truly tumor-specific antigen. Both humoral and cellular immune responses were shown to be independently associated with clinical responses following idiotype vaccination in patients with follicular lymphoma. Single arm Phase I and II idiotype vaccine trials demonstrated improved progression free survival compared with historical controls in patients with follicular lymphoma. However, data from ongoing randomized Phase III trials are needed to definitively determine the clinical benefit of idiotype vaccination in non-Hodgkin's lymphoma. If successful, idiotype vaccines are most likely to be used as an adjuvant following standard treatment with combination chemotherapy. Additionally, the recent demonstration of induction of antitumor T-cell responses by idiotype vaccination following B-cell depletion induced by rituximab[39] suggests that idiotype vaccines can be used after administration of

rituximab or rituximab-based chemotherapy. The combination of rituximab with idiotype vaccine would provide for the first time a combination biologic regimen for the treatment of this lymphoma. Indeed, the use of passively administered anti-tumor monoclonal antibodies such as rituximab with vaccines is likely to be complementary. Compared with monoclonal antibodies, vaccines are likely to target different tumor antigens, can induce immunological memory, and can induce polyclonal humoral and cellular immune responses, thereby minimizing the emergence of immune escape variants. Given that the median age of follicular lymphoma patients at diagnosis is 60 years, the development of such nontoxic immunotherapeutic approaches is highly desirable.

With the increased use of rituximab for the treatment of follicular lymphoma and other B-cell non-Hodgkin's lymphomas, further improvement in the potency of the idiotype vaccines would require strategies to enhance the T-cell responses since rituximab depletes normal B cells and impairs the generation of antibody responses following vaccination. Although novel adjuvants such as toll-like receptor ligands may prove to be more potent than cytokine adjuvants,[40-42] further improvement of the cancer vaccines would probably also require disruption of the immunoregulatory pathways that modulate the magnitude and duration of the immune response. Studies in animal models suggest that the T-cell immune responses against foreign or self-antigens are regulated by several immunoregulatory pathways and/or peripheral tolerance mechanisms.[43-45] For example, $CD4^+CD25^+$ regulatory T cells (T_{regs}) have been shown to downregulate T-cell responses against foreign antigens as well as tumor antigens, most of which are self-antigens.[46] Similarly, cytotoxic T lymphocyte-associated antigen (CTLA)-4, a molecule that is expressed on activated T cells and T_{regs}, was shown to downregulate T-cell responsiveness and prevent the initiation and/or limit the magnitude of autoreactive T-cell responses.[47] A host of other mechanisms such as programmed cell death 1 (PD-1), B7-H1, B7-H4 were recently described to negatively regulate T-cell responses.[45] These new insights have led several investigators to hypothesize that the potency of cancer vaccines can be further enhanced by concurrent suppression or blocking of peripheral tolerance mechanisms and/or suppressive immunoregulatory pathways. Thus, depletion of CD4+CD25+ T_{regs} or blockade of CTLA-4, PD-1 or B7-H1 led to improved tumor control in various murine models.[48-51] Moreover, simultaneous disruption of two immunoregulatory mechanisms by depletion of T_{regs} and blockade of CTLA-4 resulted in improved tumor rejection as compared with either one alone when used in combination with a tumor cell-based vaccine in a B16 melanoma model.[51] These preclinical observations led to the initiation of pilot clinical trials using combination immunotherapeutic strategies analogous to combination chemotherapy that has been effectively used for the curative treatment of certain cancers including lymphoma. Dannull and colleagues have recently demonstrated that vaccine-mediated antitumor immunity is significantly enhanced in renal cell cancer patients after depletion of regulatory T cells using denileukin diftitox[52] (a recombinant IL-2 diphtheria toxin conjugate; also known as Ontak); an FDA-approved drug for the treatment of cutaneous T-cell lymphomas. In another study, administration of a single dose of Ontak in ovarian cancer patients depleted T_{regs} and was associated with enhanced endogenous immunity.[53] Similarly, blockade of CTLA-4 in combination with peptide vaccination has resulted in enhanced cancer immunity and durable objective responses in patients with metastatic melanoma.[54,55] Taken together, these preclinical and early phase clinical results support the evaluation of combination immunotherapy strategies in future clinical trials with idiotype vaccination for B cell lymphoma to stimulate an antitumor T-cell response and the simultaneous suppression of immune regulatory pathways to augment the induced T-cell response. The existence of multiple immune regulatory pathways necessitates systematic evaluation of these approaches in clinical trials to determine the optimal combination immunotherapy regimen.

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EXHIBIT

AM

DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma

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Vaccination with idiotypic protein protects against B-cell lymphoma, mainly through anti-idiotypic antibody. For use in patients, DNA vaccines containing single-chain Fv derived from tumor provide a convenient alternative vaccine delivery system. However, single-chain Fv sequence alone induces low anti-idiotypic response and poor protection against lymphoma. Fusion of the gene encoding fragment C of tetanus toxin to single-chain Fv substantially promotes the anti-idiotypic response and induces strong protection against B-cell lymphoma. The same fusion design also induces protective immunity against a surface Ig-negative myeloma. These findings indicate that fusion to a pathogen sequence allows a tumor antigen to engage diverse immune mechanisms that suppress growth. This fusion design has the added advantage of overcoming potential tolerance to tumor that may exist in patients.

Idiotypic antigenic determinants are generated by recombinatorial and somatic mutational changes in the Ig variable region genes V_H and V_L, and provide ideal targets for immune attack on B-cell tumors[1,2]. In several mouse models, vaccination with idiotypic protein induces protective immunity against lymphoma, mostly mediated by anti-idiotypic antibody[2,3]. A clinical trial using this approach for low-grade follicular lymphoma is producing encouraging results[4].

One problem with widening the clinical application is that preparation of individual idiotypic proteins is technically demanding and expensive. Many alternatives are being explored, including loading idiotypic Ig onto dendritic cells[5]. However, clinical application would be greatly facilitated by the development of DNA vaccines, already found to be effective in inducing protective immunity against a range of organisms[6]. Also, DNA vaccines may activate additional immune mechanisms to attack tumor cells[7]. Initial attempts using tumor-derived V_H and V_L in a single-chain Fv (scFv) format induced only low levels of antibody and poor protective immunity in mice[8]. Protection seemed to be improved by using a vector encoding whole Ig, consisting of murine V-genes linked to human constant regions, but antibody responses were still inferior to those induced by a idiotypic protein-KLH conjugate with adjuvant[9].

To provide an 'alert' signal to the immune system, and to generate high levels of T-cell help[10], we fused a pathogen-derived gene to the scFv sequence[11]. We chose the gene encoding the fragment C (FrC) of tetanus toxin, which induces protective immunity against challenge with *Clostridium tetani*[12]. Fusion of FrC to three of three scFv sequences from patients with lymphoma led to a substantial promotion of antibody responses in mice against the patients' tumor IgM, compared with responses induced by scFv alone[11]. The antibodies seemed to be mostly anti-idiotypic, indicating optimal folding of the fused scFv (ref. 11). We report here that the fusion design is capable of inducing an anti-idiotypic response in mice that pro-

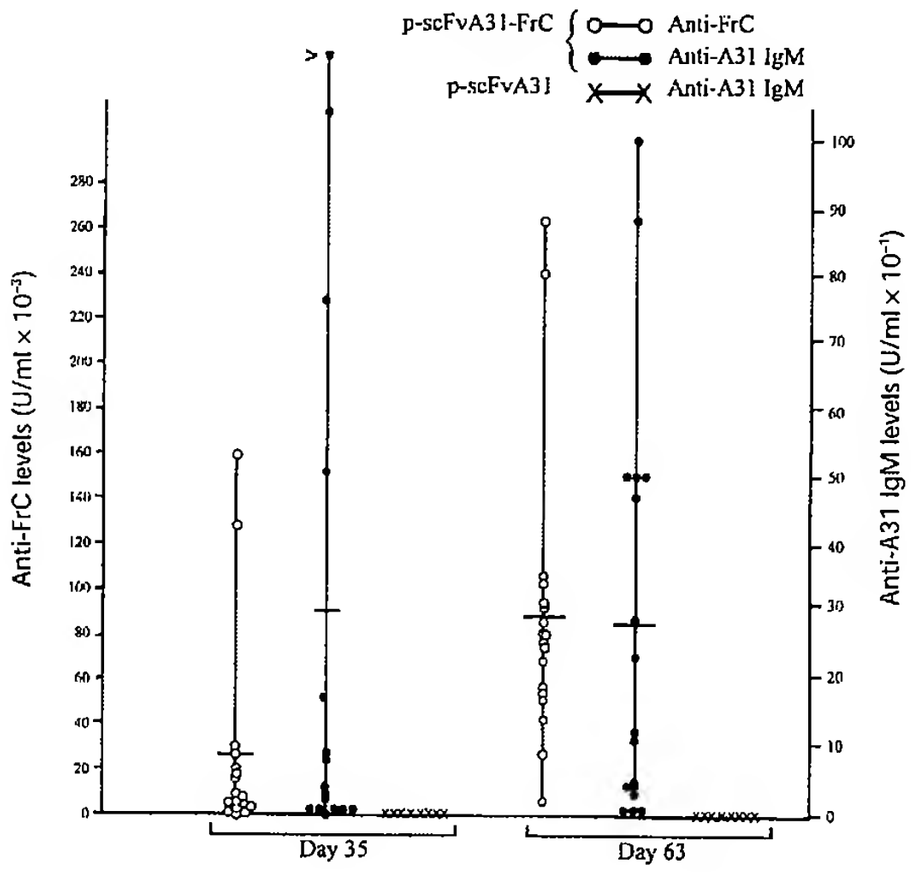

Fig. 1 Antibody responses against FrC or A31IgM induced by DNA vaccines. Vaccines contained genes encoding scFv from the A31 lymphoma, either alone (p.scFvA31) or fused to FrC (p.scFvA31–FrC). Mice were injected with 50 µg of p.scFvA31 (8 mice) or p.scFvA31–FrC (18 mice) on days 0, 21 and 42; blood samples were collected on days 35 and 63. Antibodies against FrC or against A31 IgM were measured by ELISA. Each symbol represents a value from a single mouse, with some superimposition. Mean values are indicated (short horizontal lines).

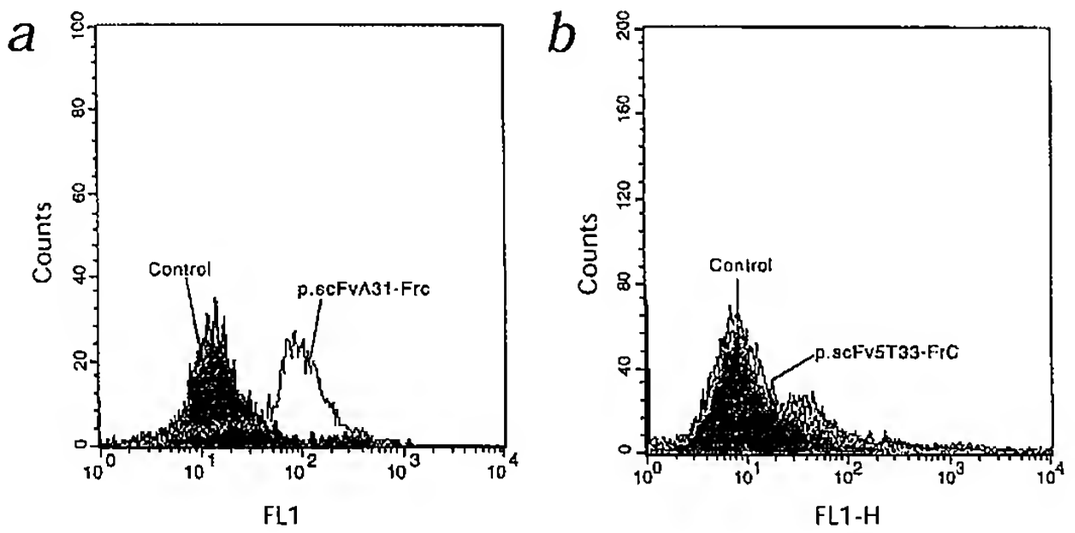

Fig. 2 Reactivity of antibodies induced by DNA fusion vaccines with target tumor cells as assessed by immunofluorescence. Pooled sera (diluted 1:10) from mice vaccinated with either (***a***) p.scFvA31–FrC or (***b***) p.scFv5T33–FrC were incubated with their target tumor cells (A31 or 5T33, respectively). Control sera were from mice vaccinated with a DNA plasmid containing a p.scFvBCL$_1$–FrC fusion gene. After incubation, cells were washed, and bound antibody detected by FITC-conjugated sheep anti-mouse Fcγ, using a FACS-Scan. The detecting antibody alone showed no significant reactivity with either A31 or 5T33 cells.

tects against challenge with syngeneic tumor. This not only was observed for a surface Ig-expressing lymphoma model but also seems to be effective against a surface Ig-negative myeloma model. This indicates that several arms of the immune response are being engaged, and raises the possibility of using DNA fusion vaccines against a wide range of B-cell tumors. Moreover, the principle of fusion vaccines may be applicable to tumor antigens of other cancers.

Results

Expression of scFv or scFv–FrC fusion genes *in vitro*

All constructs expressed protein of the expected size, as determined by the *in vitro* Promega TNT® system. To assess expression of the scFv–FrC fusion proteins, we transfected the p.scFv–FrC constructs derived from the mouse A31 lymphoma or from the 5T33 myeloma into COS-7 cells. Using an ELISA with polyclonal IgG prepared from mice vaccinated with p.FrC DNA as coating antibody, and a biotinylated version for detection, supernatants were found to contain levels of FrC protein similar to those produced from a plasmid containing the FrC gene alone (data not shown). Because the constructs all have leader sequences, with the scFv-containing constructs having their natural leader sequences, the FrC-containing protein probably arises from secretion. Similar results have been obtained with eight of eight constructs derived from patients with lymphoma and myeloma, demonstrating that the fusion gene format leads to efficient protein secretion *in vitro*.

Induction of antibody against the A31 lymphoma

Injection of a plasmid containing the A31-derived scFv fused to the FrC gene (p.scFvA31–FrC) on days 0 and 21 generated detectable antibody against idiotypic IgM from the A31 lymphoma, and against FrC, in most mice (Fig. 1). Mean levels of anti-A31Id did not increase substantially after a further injection of plasmid, but the numbers of responding mice increased. The anti-FrC response showed a definite increase in total responders and mean levels after the boost. The plasmid containing only scFv sequence (p.scFvA31) failed to induce detectable anti-A31Id antibodies after three injections. The anti-A31Id induced by the fusion gene showed no reactivity by ELISA with IgM protein from the BCL$_1$ lymphoma[2], or with Fabγ from the 5T33 myeloma IgG (not shown). Pooled immune serum reacted specifically with A31 tumor cells by FACS analysis (Fig. 2*a*). As an additional control, pooled immune serum was tested against cells from the mouse BCL$_1$ lymphoma[2], with completely negative results (not shown).

Induction of antibody against the 5T33 myeloma

Injection of a single dose of plasmid containing the 5T33-derived scFv sequence fused to the FrC gene (p.scFv5T33–FrC) led to a rapid antibody response against idiotypic Fab from the 5T33 tumor, and against FrC (Fig. 3). Serum levels of anti-FrC and anti-5T33Id antibodies varied, but were evident in all mice at day 21. In contrast, the plasmid containing only scFv sequence (p.scFv5T33) induced no detectable anti-5T33Id antibody. A second injection (on day 21) did not change the levels of antibodies substantially, but the third injection on day 42 increased antibody levels induced by the p.scFv5T33–FrC fusion construct, and led to a very low response using p.scFv5T33 alone (Fig. 3). Pooled immune serum reacted specifically with 5T33 Fab by ELISA, with no reactivity with Fab from an unrelated mouse IgG monoclonal antibody, or with A31IgM or BCL$_1$IgM (not shown). No reactivity with 5T33 cell surface was detectable by FACS analysis (Fig.2b), consistent with the reported low or absent surface Ig on the 5T33 myeloma cells[13]. This was confirmed by the negative results using FITC-conjugated sheep anti-Fcγ antibody alone (not shown).

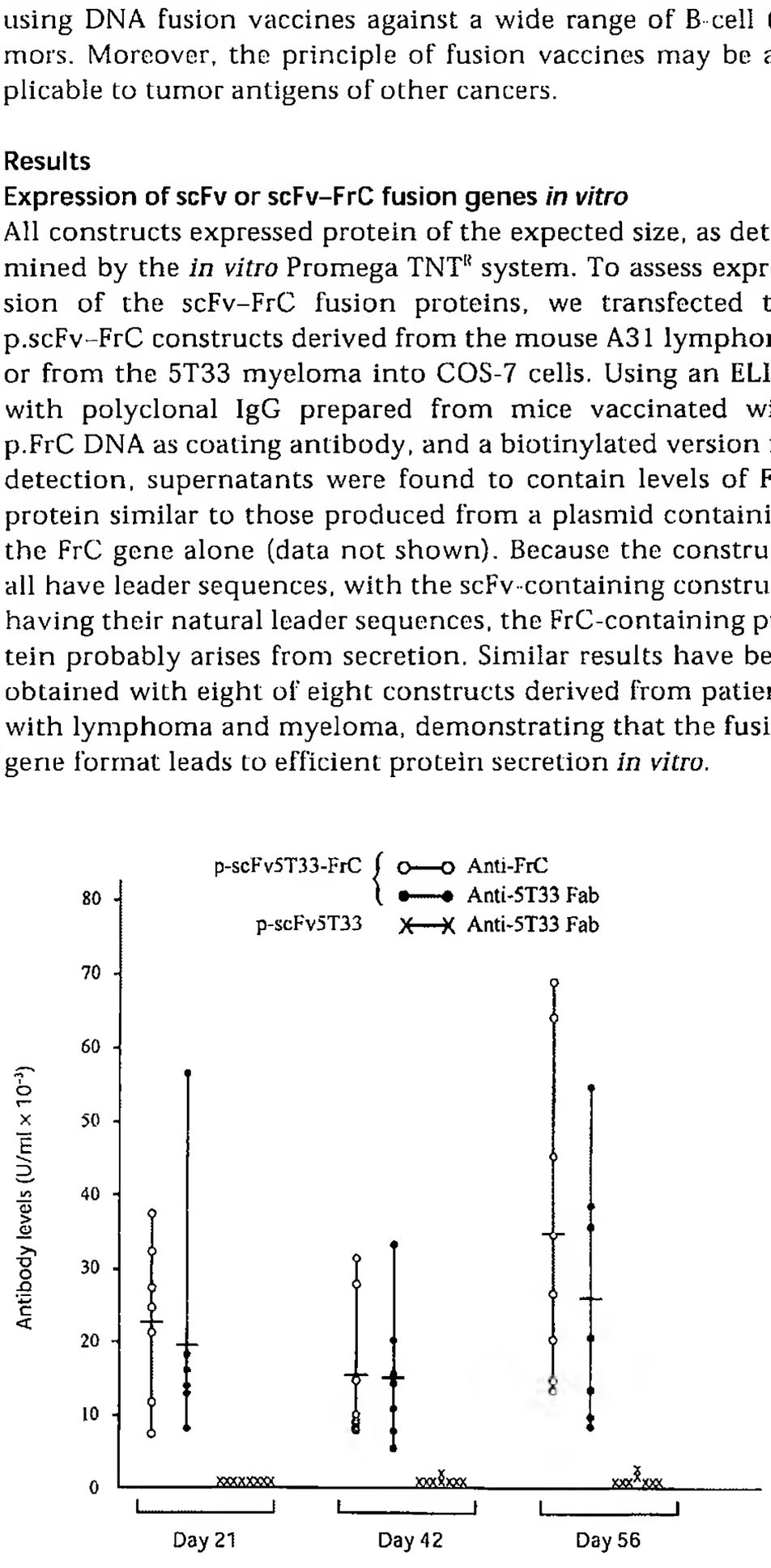

Fig. 3 Antibody responses against FrC or against 5T33Fab induced by DNA vaccines. Vaccines contained genes encoding scFv from the 5T33 myeloma, either alone (p.scFv5T33), or fused to FrC (p.scFv5T33–FrC). Mice (8 per group) were injected with 50 μg of plasmid on days 0, 21 and 42; blood samples were taken before the second and third injections, and on day 56. Antibodies against FrC or against 5T33Fab were measured by ELISA. Each symbol represents a value from a single mouse, with some superimposition. Mean values are indicated (short horizontal lines).

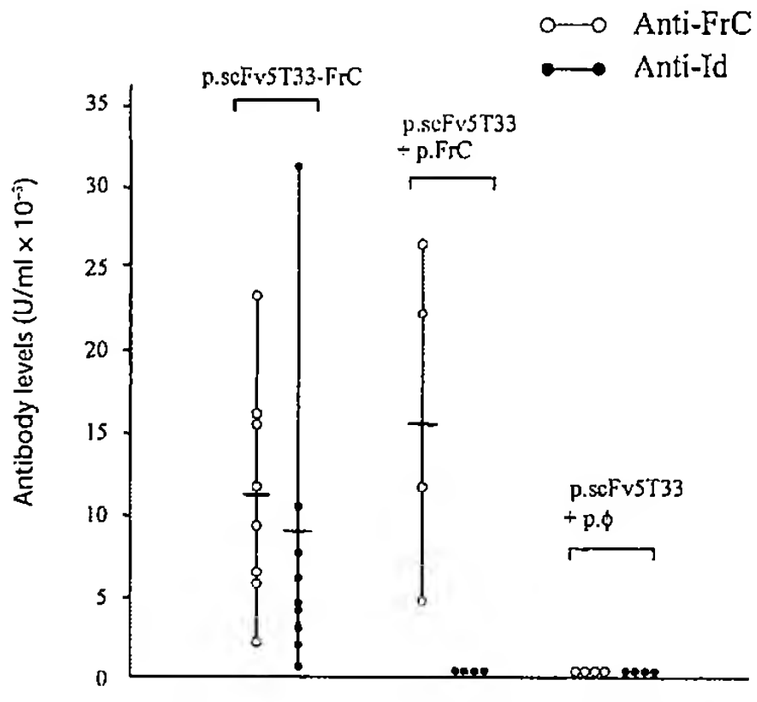

Fig. 4 Antibody responses against 5T33Fab induced by DNA vaccines requires gene fusion. Mice were injected on day 0 with 50 μg of fusion construct (p.scFv5T33–FrC), or 50 μg each of separate vectors p.scFv and p.FrC, or 50 μg p.scFv and p.Φ empty vector mixed together. Blood samples were taken on day 21, and antibodies against FrC or against 5T33Fab were measured by ELISA. Each symbol represents a value from a single mouse. Mean values are indicated (short horizontal lines).

Requirement for fusion of scFv sequence to FrC

We determined whether fusion of scFv to FrC was required in the 5T33 model by comparing induction of anti-5T33Id by the fusion construct with that induced by the p.scFv5T33 and p.FrC constructs injected together as separate vectors. Antibody responses at day 21 showed that, although the mixed plasmids induced anti-FrC antibodies at levels comparable with those from the fusion construct, there was no detectable anti-5T33Id antibody (Fig. 4). This pattern was maintained after two more injections. In fact, the separate p.FrC vector was as ineffective as empty vector in promoting a response to p.scFv (Fig. 4). This confirmed that p.FrC was capable of inducing anti-FrC antibodies as expected, but that fusion was required for induction of antibodies against the scFv component.

Profile of Ig subclasses in induced antibodies

The IgG subclass profile of the antibodies induced by p.scFvA31–FrC in CBA/HxC57BL6 F1 mice or by p.scFv5T33–FrC in C57BLKalwRij mice indicated dominance of IgG1 in most antibody populations (Table). However, IgG2a was detectable, and was dominant in the anti-FrC response to p.scFvA31–FrC. Comparison with antibodies induced by idiotypic protein/CFA showed in both models that total IgG antibody levels were higher with protein/CFA than with DNA vaccination, although responses in the 5T33 model were in the same range. However, antibodies induced by protein/CFA were almost entirely of the IgG1 subclass in both cases (Table). These results confirm that intramuscular vaccination with DNA will activate a T_H1 response[6,11], but show that the dominant anti-Id response is consistent with activation of T_H2 cells.

Induction of cytotoxic T cells

One goal in attacking surface Ig-negative myeloma cells is to activate a cytotoxic T-lymphocyte (CTL) response. This was sought using splenocytes collected at day 35 from mice vaccinated with p.scFv5T33-FrC. Because there is no obvious candidate peptide for binding to H-2D^b or H-2K^b in the 5T33 unique sequence[14], we used cells from the 5T33 *in vitro* line for re-stimulation and as targets. No cytotoxicity was observed (Fig. 5). In contrast, using the same splenocytes with a candidate H-2K^b-binding peptide from FrC (SNWYFNHL), we detected definite CTL activity against peptide-loaded EL-4 target cells. The 5T33 *in vitro* line could also be killed when loaded with the FrC peptide (Fig.5). It seems, therefore, that vaccination with p.scFv5T33–FrC induces CTLs against FrC, but not against scFv5T33, consistent with a lack of a peptide motif in the scFv sequence.

Induction of protective immunity against the A31 lymphoma

Vaccination with the fusion gene p.scFvA31–FrC induced strong protection against challenge with A31 lymphoma (Fig. 6), with $P < 0.01$ (log rank test) compared with non-vaccinated control mice. In contrast, p.scFvA31 alone was ineffective. Mice vaccinated with a control construct containing scFv from the BCL_1 lymphoma fused to FrC (p.scFvBCL_1–FrC) were also not protected. In fact, mice in this group seemed to survive less well than the non-vaccinated control mice, although this did not reach statistical significance (Fig. 6). These results show that protection against the A31 lymphoma requires fusion of the tumor-derived scFv to FrC, and that neither component alone is effective.

Induction of protective immunity against the 5T33 myeloma

Vaccination with the fusion gene p.scFv5T33–FrC induced definite protection against challenge with 5T33 myeloma (Fig. 7), with $P < 0.01$ compared with non-vaccinated control mice. In contrast, vaccination with p.scFv5T33 alone was ineffective. Because the 5T33 myeloma cells express no surface IgG, and the immune serum showed the expected failure to bind to 5T33 cells (Fig. 2*b*), it seemed that antibody was unlikely to be protective. To assess this, mice were vaccinated with 5T33 IgG protein in CFA, which induced high levels of anti-idiotypic antibody (Table). However, these vaccinated mice showed no protection against challenge (Fig. 7), with survival indistinguishable from mice vaccinated with CFA alone. In fact, mice vaccinated with CFA or 5T33 protein/CFA survived less well than non-vaccinated mice (Fig.7). These results indicate that protection induced by p.scFv5T33–FrC is unlikely to be antibody-mediated.

Effect of pre-existing anti-FrC antibody

In patients, a scFv–FrC fusion vaccine probably will have to be administered in the presence of pre-existing antibody against tetanus toxoid (TT). To simulate this, we pre-vaccinated eight mice at day –21 with a conventional TT vaccine in aluminum

Table Isotypes of anitbody responses against idiotypic Ig or Fragment C

Vaccine	Ag	Ab IgG subclass IgG1 (U/ml)	IgG2a (U/ml)	Ratio IgG1:IgG2a
p.scFvA31–FrC	A31IgM	2,100	120	18:1
	FrC	7,600	19,200	0.4:1
A31IgM/CFA	A31IgM	192,000	1,400	137:1
p.scFv5T33–FrC	5T33Fab	34,000	1,200	28:1
	FrC	59,200	2,700	22:1
5T33IgG/CFA	5T33Fab	90,000	0	∞

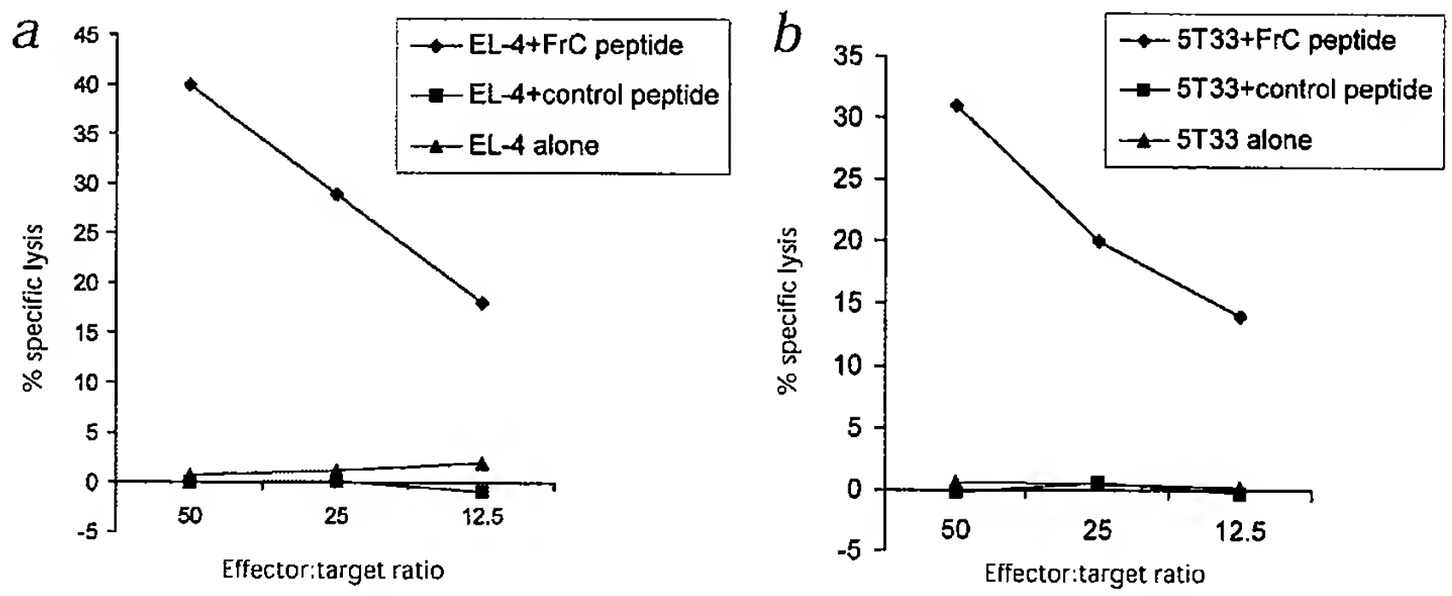

Fig. 5 Induction of CTLs against FrC by the DNA fusion vaccine. Mice (4 per group) were injected with p.scFv5T33–FrC on days 0 and 21. Splenocytes were collected on day 35 and, after re-stimulation *in vitro* with irradiated 5T33 cells or with normal splenocytes 'pulsed' with the SNWYFNHL peptide from FrC, were tested for CTL activity against EL-4 cells (***a***) or 5T33 cells (***b***), each loaded with test peptide or control peptide from influenza virus (ASNENDAM).

hydroxide (alum). This induced high levels of anti-FrC antibodies (median value, 9,000 U/ml). We then vaccinated these mice with the p.scFv–FrC construct from the 5T33 myeloma and assessed induction of antibodies against the 5T33Id. Comparison was made with mice pre-vaccinated with a control antigen in alum, and with mice that were not pre-vaccinated. Anti-5T33Id antibodies were induced in all pre-vaccinated mice, with no significant difference between levels in those pre-vaccinated with control antigen or with TT (Fig. 8)($P > 0.09$, Mann-Whitney non-parametric test). This demonstrates that pre-existing antibody against FrC has not inhibited induction of anti-idiotypic responses. Pre-vaccination with alum itself seems to have given a small 'boost' to the subsequent response to p.scFv–FrC (Fig. 8).

Discussion

In follicular lymphoma and myeloma, it is possible to bring patients into clinical remission. However, it is difficult to eradicate all tumor cells, and eventual relapse is common. Vaccination could be a useful intervention during remission to mobilize the immune system against residual disease. In this context, idiotypic proteins are known candidate tumor antigens[1,2]. The DNA scFv format offers ease of construction and manipulation to widen the approach of idiotypic vaccination. However, injection into muscle, with subsequent synthesis of scFv protein, is insufficient to engage the immune response[8].

Fusion to a pathogen sequence, such as FrC of TT, overcomes this problem, and leads to high levels of antibody against both the tumor Ig and FrC (ref. 11). We have found the same promotional activity of fusion to FrC for scFv constructs from three of three patients with lymphoma[11] and four of four patients with myeloma (C.A.F., *et al.*, manuscript in preparation). In each case, anti-idiotypic antibodies were induced in mice, indicating optimal folding of the scFv. The mechanism of promotion is likely to include activation of anti-FrC T-cell help, as described for peptides linked to TT (ref. 10). The concept of cognate T-cell help is supported by the evident requirement for fusion of the scFv and FrC genes. Mobilization of these T cells during vaccination of patients should also overcome the potential problem of tolerance in $CD4^+$ T cells that may occur after long-term exposure to idiotypic protein[15].

The mechanism of protection against the A31 lymphoma is likely to be antibody, as demonstrated after vaccination with idiotypic protein/CFA (ref. 16). Lymphoma cells seem sensitive to suppression *in vivo* by polyclonal anti-idiotypic antibodies, possibly due to direct induction of apoptosis[17]. In contrast, it is very unlikely that antibody has a protective role against the 5T33 myeloma. The absence of surface Ig, and the failure of the high levels of anti-idiotypic antibodies induced by vaccination with idiotypic protein to protect against 5T33 challenge, both argue against a role for antibody.

It is more difficult to assess the role of $CD4^+$ T cells in protection. Proliferative responses to FrC after vaccination with the fusion constructs were detectable in both models. However, proliferative responses to idiotypic Ig were insignificant (data not shown). The low proliferative response to idiotypic Ig closely resembles that seen in mice vaccinated with idiotypic IgM/CFA (ref. 18), and may reflect poor uptake and presentation by splenic antigen presenting cells *in vitro*. Because there is no candidate CTL motif in the scFv of 5T33, and no evidence for CTL activity, it remains likely that $CD4^+$ T cells are the mediators of protection. The mechanism may be similar to that described using transgenic $CD4^+$ T cells specific for a light chain idiotype[15]. These cells alone were able to suppress growth of an

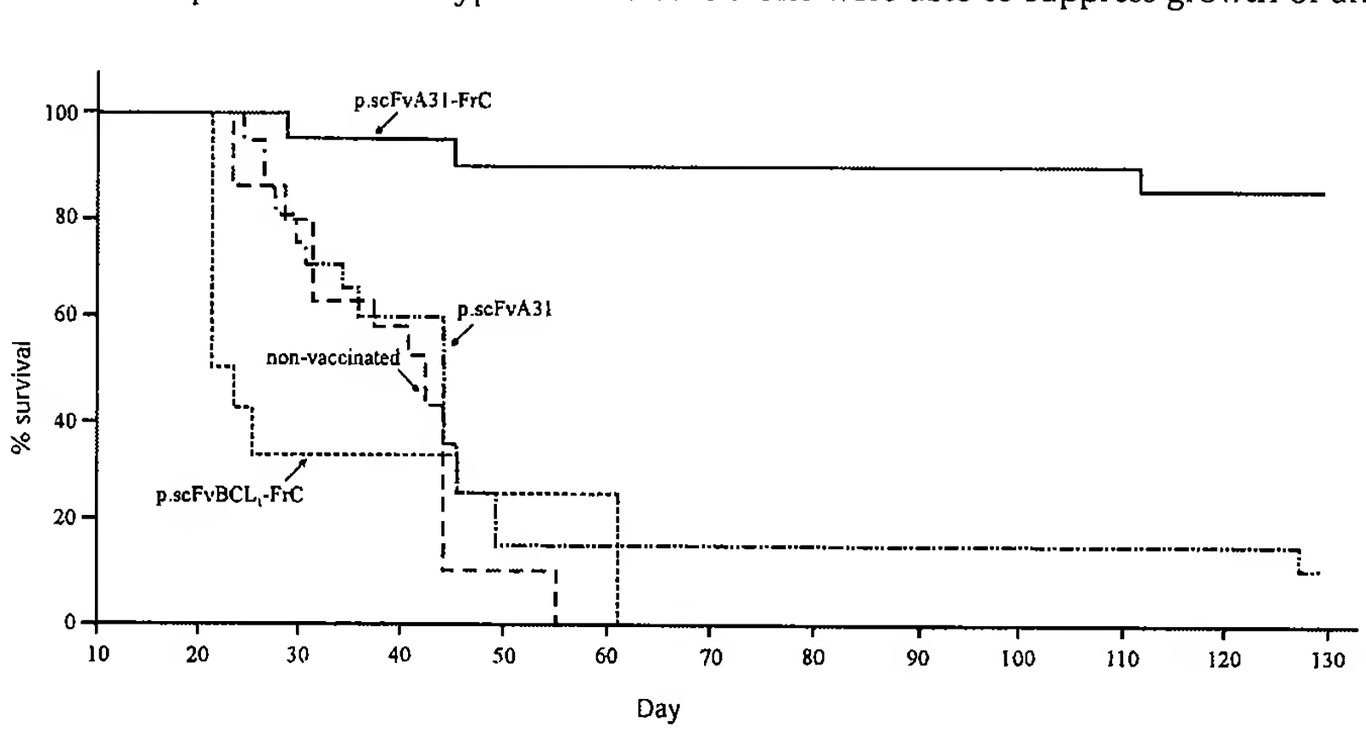

Fig. 6 Induction of protective immunity against the A31 lymphoma by the DNA fusion vaccine. Mice were injected with 50 µg of p.scFvA31 (20 mice), p.scFvA31–FrC fusion construct (20 mice), or the control fusion construct p.scFvBCL$_1$–FrC (9 mice) on days 0, 21 and 42. They were then challenged with 10^4 A31 cells and their survival was compared with that of non-vaccinated (n = 12) mice.

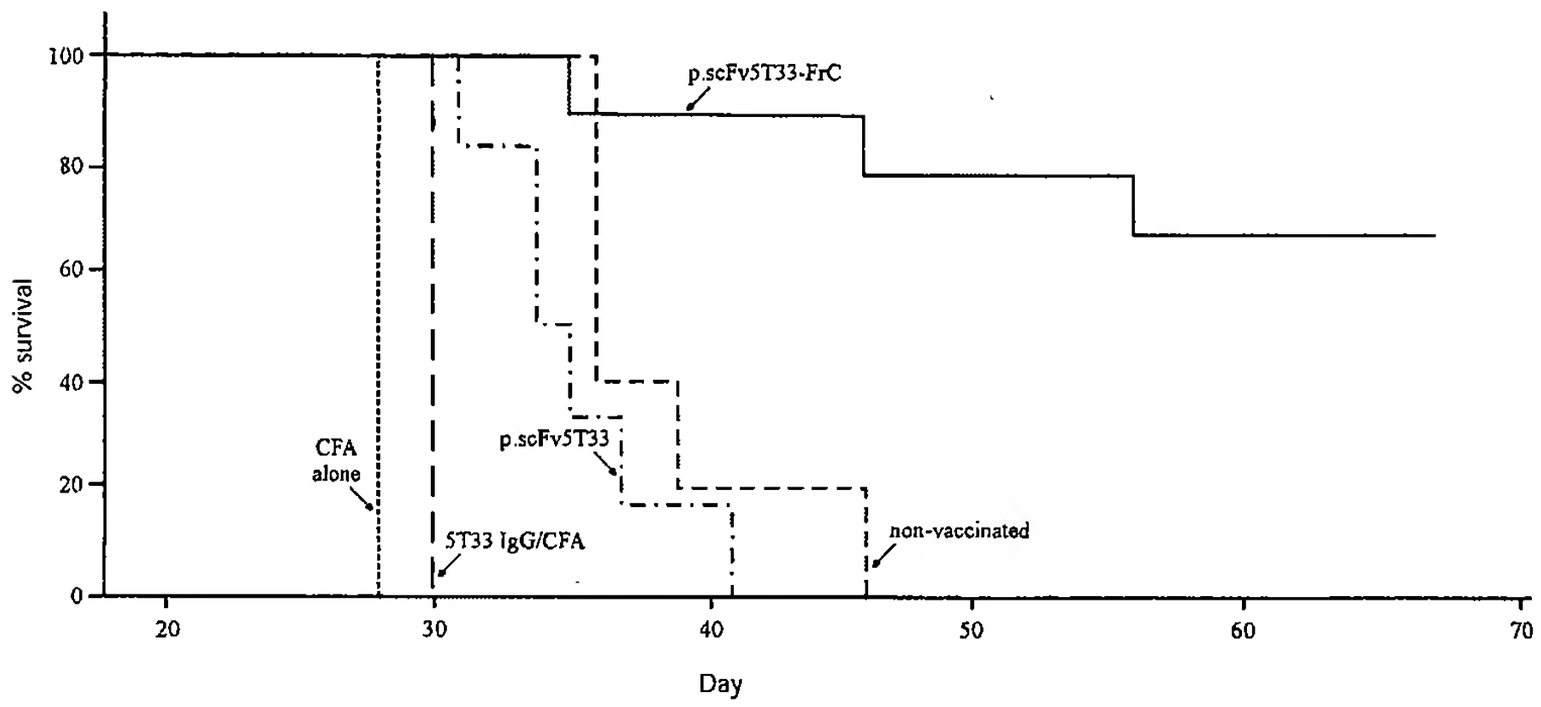

Fig. 7 Induction of protective immunity against the 5T33 myeloma by the DNA fusion vaccine. Mice were injected with 50 µg of p.scFv5T33 (6 mice) or p.scFv5T33–FrC (10 mice) on days 0, 21 and 42. Separate groups of 4 mice were injected with 5T33IgG/CFA, or CFA alone on days 0, 21 and 28. They were than challenged with 1×10^4 5T33 cells and their survival was compared with that of non-vaccinated ($n = 10$) mice.

MHC class II-negative plasmacytoma by a pathway involving processing and presentation of secreted myeloma protein by host antigen presenting cells. The mediators of suppression probably are released cytokines[15]. We are seeking these cells in our vaccinated mice using dendritic cells as alternative antigen presenting cells. Although the details of the mechanisms are not yet understood, the ability of DNA vaccines containing fused scFv–FrC genes to induce protection against challenge will encourage the planned clinical trial against lymphoma[19], and stimulate an extension of the approach to myeloma. Although patients are likely to have pre-existing antibodies to TT, this seems to have no substantial influence on the ability to induce anti-idiotypic responses by the DNA vaccine route.

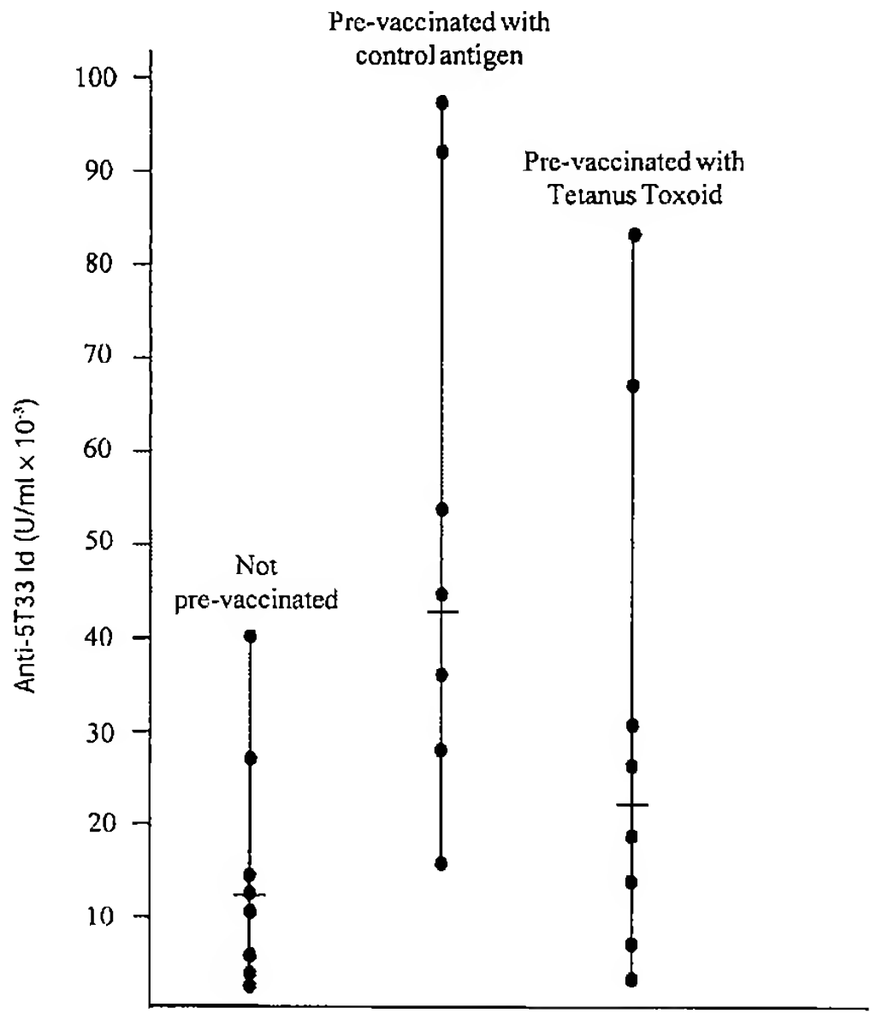

Fig. 8 Effect of pre-vaccination with tetanus toxoid on induction of anti-Id antibodies by the p.scFv5T33–FrC construct. Three groups of mice were used: one group was pre-vaccinated with TT adsorbed to alum; a second group was pre-vaccinated with a control alum-adsorbed antigen, nucleoprotein of influenza virus; and a third group was not pre-vaccinated. Pre-vaccination was on day –21 before vaccination of all groups with the p.scFv5T33–FrC construct on days 0, 21 and 42. Blood samples were collected on day 63 for measurement of anti-5T33Id antibodies. Each symbol represents an individual mouse and the median levels of each group are indicated (short horizontal lines).

Methods

Mouse tumors and idiotypic proteins. The A31 B-cell splenic lymphoma was passaged in CBA/HxC57BL6 F1 mice by intravenous injection[16]. Idiotypic IgMκ was prepared from the supernatant of an A31/X-63.Ag8.653 hybridoma by dialysis into water[20], and was detected with the anti-idiotypic monoclonal antibody[16]. The 5T33 *in vitro* myeloma cell line[13] was passaged in C57BL/KaLwRij mice by intraperitoneal injection. Idiotypic IgG2b was purified from ascitic fluid by immunoabsorption on protein G, and the Fabγ fragment was prepared by digestion with pepsin.

DNA vaccine constructs. The V_H and V_L sequences of the A31 lymphoma and the 5T33 myeloma were obtained by PCR amplification with a consensus framework 1 and mixed J_H primers[21] for V_H, and with a framework 1 primer and a Cκ primer[22] for Vκ, followed by cloning and sequencing. Sequences have been deposited in the EMBL database, with accession numbers AJ006791 and AJ006792 for A31 V_H and Vκ; and AJ0066946 and AJ006947 for 5T33 V_H and Vκ. The A31 V_H and 5T33 Vκ were in germ-line configuration, and the A31 Vκ and 5T33 V_H were identical to published rearranged genes. V_H genes of A31 and 5T33 tumors were re-amplified with the leader primers specific for A31 and 5T33 which also contained the *Hin*dIII restriction site to facilitate cloning[11]. Primer sequences, with cloning sites underlined, were A31V_H leader, 5'–TATAAGCTTGCCGCCACCATGAAGTTGTGGCTGACC–3'; and 5T33V_H leader, 5'–TATAAGCTTGCCGCCACCATGGAATGGAGCAGAGTC–3'. V_H and V_L of A31 and 5T33 tumors were assembled as scFv or scFv–FrC as described[11]. Assembled genes were cloned into the expression vector pcDNA3 (InvitroGen BV, Leek, The Netherlands) as *Hin*dIII–*Not*I fragments and plasmid DNA purified for vaccination using a GigaPrep kit (Qiagen, Hilden, Germany).

Vaccination and tumor challenge. Vaccination with DNA vaccines containing scFv or scFv–FrC genes was done by injecting 50 µg of DNA into two sites in the quadriceps muscles on days 0, 21 and 42 (ref. 11) for both models (A31 and 5T33). Tail blood was collected before injection and in some cases on day 63 before tumor challenge. Vaccination with idiotypic Ig (50 µg) in CFA was by subcutaneous injection on days 0, 21 and 28, with tail blood collected before tumor challenge[2,16]. Challenge was on day 63 with 1×10^4 A31 or 5T33 cells injected intravenously. Mice were culled according to the recommended Humane End Point procedure (UK Coordinating Committee on Cancer Research, London, England).

Pre-vaccination of mice with TT was done using 8 IU of TT adsorbed to aluminium hydroxide (alum)(Evans Medical, Leatherhead, England). The control antigen was nucleoprotein of influenza virus A/NT/60/68 (50 µg) (provided by R. Gonsalves, National Institue for Medical Research, Mill Hill, London, England), also adsorbed to alum. Each adsorbed antigen was injected into two subcutaneous sites 21 days before vaccinating with the

DNA scFv–FrC vaccine construct.

Measurement of antibody reponses. Anti-idiotypic antibodies against A31IgM or 5T33IgG were measured by ELISA (ref. 16). Idiotypic A31 IgM or 5T33Fab were coated at 500 ng/ml and, after exposure to diluted control or immune sera, bound mouse IgG were detected by HRP-sheep anti-mouse Fcγ (The Binding Site, Birmingham, England). Units/ml were measured using arbitrary standards[16]. Controls included IgM from the BCL_1 lymphoma[2] and Fabγ from OKT3 (American Type Culture Collection). IgG subclasses were measured by ELISA, with HRP-rabbit anti mouse IgG1 or HRP-goat anti-mouse IgG2a as detecting antibodies (Harlan-Seralab, Crawley, West Sussex, England). Binding to A31, 5T33, or BCL_1 cells was assessed by FACS-Scan, using sera diluted 1:10 with FITC-conjugated sheep anti-mouse Fcγ-detecting antibody (Sigma).

Cytotoxicity assay. Spleens were taken from vaccinated mice at day 56 and lymphocytes suspended at 10^6/ml. Normal splenocytes 'pulsed' with 10 μM FrC peptide (SNWYFNHL) were irradiated and incubated with the CTLs for 7 days with human rIL-2. This was repeated and cells were then incubated for 4 h with ^{51}Cr-labelled target EL-4 cells pre-incubated with peptide. Released radioactivity was measured and specific lysis was calculated. For assessment of CTL activity against 5T33 cells, irradiated (2500 R) 5T33 cells (10^6/ml) were used for restimulation with rIL-2. For assay, 5T33 cells, either alone or pre-pulsed with peptide, were used as described for EL-4 cells.

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EXHIBIT

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Phase 1 Safety and Immunogenicity Evaluation of a Multiclade HIV-1 DNA Candidate Vaccine

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Abstract

Background—Gene-based vaccine delivery is an important strategy in the development of a preventive vaccine for acquired immunodeficiency syndrome (AIDS). Vaccine Research Center (VRC) 004 is the first phase 1 dose-escalation study of a multiclade HIV-1 DNA vaccine.

Methods—VRC-HIVDNA009–00-VP is a 4-plasmid mixture encoding subtype B Gag-Pol-Nef fusion protein and modified envelope (Env) constructs from subtypes A, B, and C. Fifty healthy, uninfected adults were randomized to receive either placebo ($n = 10$) or study vaccine at 2 mg ($n = 5$), 4 mg ($n = 20$), or 8 mg ($n = 15$) by needle-free intramuscular injection. Humoral responses (measured by enzyme-linked immunosorbant assay, Western blotting, and neutralization assay) and T cell responses (measured by enzyme-linked immunospot assay and intracellular cytokine staining after stimulation with antigen-specific peptide pools) were measured.

Results—The vaccine was well tolerated and induced cellular and humoral responses. The maximal $CD4^+$ and $CD8^+$ T cell responses occurred after 3 injections and were in response to Env peptide pools. The pattern of cytokine expression by vaccine-induced HIV-specific T cells evolved over time, with a diminished frequency of interferon-γ–producing T cells and an increased frequency of interleukin-2–producing T cells at 1 year.

Conclusions—DNA vaccination induced antibody to and T cell responses against 3 major HIV-1 subtypes and will be further evaluated as a potential component of a preventive AIDS vaccine regimen.

More than 25 million people have died since HIV/AIDS was identified in 1981, and an estimated 14,000 new infections occur daily [1]. Development of a globally relevant HIV-1 vaccine is critical for controlling this pandemic. The combination of a high transcriptional error rate and frequent recombination results in a remarkable amount of genetic diversity among HIV-1 strains and presents a challenge for selecting vaccine antigens. A joint meeting between the Vaccine Research Center (VRC) (National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH], Department of Health and Human Services) and

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[a]Study group members are listed after the text

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the Joint United Nations Programme on HIV/AIDS concluded that testing of multiclade candidate vaccines is a high international scientific priority [2]. Sequences encoding gene products from 3 high-incident HIV sub-types were used to produce the candidate vaccine evaluated in the present study [3,4]. Multiple antigens expressed by the vaccine elicit responses against multiple epitopes and may diminish the chances for immune escape.

Delivering antigens by DNA plasmids has potential advantages over other vector delivery systems, notably the lack of antivector immunity. Despite examples of vaccine-induced protection in mice and nonhuman primates [5,6], DNA immunization has shown limited immunogenicity in humans [7–11]. Here, we report the findings from a phase 1 clinical trial of a multigene, multiclade HIV-1 DNA candidate vaccine and demonstrate the induction of HIV-1–specific T cell and antibody responses.

METHODS

Study design

VRC 004 (NIH 03-I-0022) was conducted at the NIH by the VRC. It was a randomized, double-blind, placebo-controlled phase 1 dose-escalation study. A randomization sequence was obtained by use of computer-generated random numbers, and blocking ensured balance across groups. The study was opened to accrual on 13 November 2002 and was unblinded on 24 September 2004. Vaccine safety and immunogenicity were the primary and secondary objectives, respectively. Eligibility criteria included HIV-seronegative status; age 18–40 years; being amenable to HIV risk-reduction counseling; having good general health as determined by medical history, physical exam, and laboratory tests; and having no prior exposure to investigational HIV vaccines. Fifty subjects were randomized to receive placebo ($n = 10$) or vaccine at doses of 2 mg ($n = 5$), 4 mg ($n = 20$), or 8 mg ($n = 15$). Safety reviews were conducted in both the 2-mg and 4-mg groups (5 vaccine recipients and 2 placebo recipients) before randomizing the remaining 36 subjects to the 4-mg, 8-mg, or placebo groups. The NIAID Intramural Data and Safety Monitoring Board conducted safety reviews for the dose escalation from 4 to 8 mg as well as at 6-month intervals throughout the study. Injections (1 mL/injection) were administered on day 0 and at weeks 4 and 8. Arms were alternated for sequential vaccinations, except for the delivery of the 8-mg dose of vaccine, which required 1-mL injections of 4 mg into both arms. Evaluations included laboratory tests, physical assessments by clinicians, and self-assessment for local and systemic symptoms recorded on 7-day diary cards. Adverse events were graded for severity by use of a preapproved table that incorporated a 5-point scale and were coded by use of Medical Dictionary for Regulatory Activities terminology. HIV testing was done by RNA polymerase chain reaction (Roche Amplicor HIV-1 Monitor Test) and ELISA (Abbott HIVAB HIV-1/HIV-2 rDNA); Western blotting (Genetic Systems HIV Western blot kit; BioRad Laboratories; performed at the Mayo Laboratory, Rochester, MN) was done if ELISA results were positive. The social impact of participating in an HIV vaccine study was monitored.

Vaccine

The vaccine, VRC-HIVDNA009–00-VP, was developed by the VRC and is manufactured by Vical; it is composed of 4 closed, circular, DNA plasmids at a concentration of either 2 mg/mL or 4 mg/mL (figure 1). The plasmid expressing clade B HIV-1 Gag-Pol-Nef fusion polyproteins comprised 50% of the vaccine by weight. The plasmids expressing Env glycoprotein from clades A, B, and C each comprised 16.67% of the vaccine by weight. Before formulation of the vaccine product, expression levels of individual plasmids were assessed semiquantitatively by Western blot densitometry and were compared with standards run under the same conditions. Preclinical testing demonstrated the product to have an acceptable safety profile [13,14].

The placebo used was calcium and magnesium free (Vical). All injections were administered with a needle-free injection device into the deltoid muscle by use of a Biojector 2000 (Bioject).

Antibodies

Unconjugated mouse anti–human CD28, unconjugated mouse anti–human CD49d, allophycocyanin (APC)–conjugated mouse anti–human CD3, fluorescein isothiocyanate (FITC)–conjugated mouse anti–human CD8, peridinin chlorophyll protein–conjugated mouse anti–human CD4, and a mixture of phycoerythrin (PE)–conjugated mouse anti–human interferon (IFN)–γ and interleukin (IL)–2 monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS). Independent evaluation of IFN-γ and IL-2 required CD4cy5.5PE and CD8QDot655 conjugation (for details, see http://drmr.com/abcon) in combination with CD3cy7APC, IFN-γ FITC, and IL-2 APC (BDIS). To determine the optimum concentration for staining, reagents were independently titrated.

Cell preparation

Peripheral-blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque density gradient centrifugation (Pharmacia). PBMCs were frozen in heat-inactivated fetal calf serum containing 10% dimethylsulfoxide in a Forma CryoMed cell freezer. Cells were stored at −180°C. Immunogenicity assays were performed on thawed specimens; average viability was >95%.

Peptides

Peptides (15 aa in length, overlapping by 11 aa, and corresponding to the vaccine inserts) were synthesized at >85% purity as confirmed by high-performance liquid chromatography. Peptides were pooled for each protein (EnvA, EnvB, EnvC, Gag, Nef, and Pol [2 pools]) and were used at a final concentration of 2.5 μg/mL.

Cell stimulation

PBMCs (10^6) in 200 μL of R-10 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin sulfate, and 1.7 mmol/L sodium glutamate) were incubated with costimulatory anti-CD28 and anti-CD49d monoclonal antibodies (1 μg/mL each) each and with each peptide (2.5 μg/ mL) in 96-well V-bottom plates. Cells incubated with costimulatory antibodies only were included in every experiment, to control for spontaneous production of cytokine and activation of cells before addition of peptides. Staphylococcal enterotoxin B (SEB; 1 μg/mL; Sigma-Aldrich) was used as a positive control for lymphocyte activation. Cultures were incubated at 37°C in a 5% CO_2 incubator for 6 h in the presence of brefeldin A (10 μg/mL; Sigma).

Intracellular cytokine staining (ICS) assays

ICS assays for IFN-γ and/or IL-2 in $CD4^+$ and $CD8^+$ T cells were performed after stimulation with overlapping peptide pools representing the vaccine antigens. PBMCs were permeabilized for 7 min in 200 μL of a solution containing 100 μL of Tween 20 (Sigma), 160 μL of deionized water, and 40 μL of 10× FACS-Lyse solution (BDIS) at room temperature; were washed twice in cold Dulbecco's PBS containing 1% fetal bovine serum and 0.02% sodium azide (FACS buffer); and were stained with directly conjugated anti-human antibodies for 20 min on ice. Stained cells were then immediately washed twice with cold FACS buffer. The cells were resuspended in Dulbecco's PBS containing 1% paraformaldehyde (Electron Microscopy Systems) and then stored at 4°C until analysis.

Flow-cytometric analysis

Flow-cytometric analysis was performed on a FACSCalibur flow cytometer (BDIS). Between 50,000 and 250,000 events, gated on small lymphocytes, were acquired and analyzed using FlowJo software (version 8.1; Tree Star Software). The same cytokine, CD4, and CD8 gates were used for the entire trial.

Enzyme-linked immunospot (ELISpot) assays

ELISpot assays were performed using a commercially available ELISpot kit (BD Biosciences) [3]. PBMCs were stimulated overnight at 37°C in triplicate wells at a density of 2×10^5 cells/well for all stimulations other than SEB, which was conducted at 5×10^4 cells/well. After incubation, cells were lysed, and the wells were washed and incubated for 2 h at room temperature in the presence of biotinylated IFN-γ detection antibodies. Subsequently, the wells were incubated with an avidin–horseradish peroxidase solution for 1 h at room temperature, followed by incubation for 20 min with 3-amino-9-ethylcarbazole substrate solution. The plate was air-dried for a minimum of 2 h before spot quantitation on a CTL ELISpot image analyzer (Cellular Technology). Results are expressed as the mean number of spot-forming cells per 10^6 PBMCs.

Measurement of antibody responses

Research ELISAs were performed to delineate the antibody response to individual viral antigens encoded in the vaccine. End-point titers of antibodies directed against HIV antigens were determined using 96-well Immulon 2 plates (Dynex Technologies) coated with a preparation of purified recombinant HIV proteins, in accordance with methods adapted from those described elsewhere [15]. Analysis by immunoprecipitation followed by Western blotting (IP–Western blotting) was performed as described elsewhere [3]. Screening for HIV-1 neutralization activity was performed on 1:5 dilutions of serum in a flow cytometry–based assay that measured levels of intracellular p24 after a single round of HIV-1 infection of PBMCs, as described elsewhere [16].

Statistical methods

T cell data are summarized by rates of positive response to the individual peptide pools and exact, 2-sided 95% confidence intervals (CIs). Positivity criteria consisted of a statistical hypothesis test for a difference between stimulated and unstimulated wells and a minimal level of response requirement, as described elsewhere [17]. The minimum threshold for background-corrected positive-response percentage was 0.0241% for $CD4^+$ T cells and 0.0445% for $CD8^+$ T cells for the ICS assays and was 50 sfc/10^6 PBMCs for the ELISpot assays. SAS (version 8.2; SAS Institute) and Splus (version 6.0; Insightful) were used for analyses.

RESULTS

Subject population

Subjects had a mean age of 29 years, and 60% were men. Vaccine and placebo recipients had similar demographic characteristics (table 1). Vaccinations were completed in 9 of the 10 subjects in the placebo group, in 5 of the 5 subjects in the 2-mg group, in 20 of the 20 subjects in the 4-mg group, and in 14 of the 15 subjects in the 8-mg group. All subjects completed 52 weeks of follow-up.

Vaccine safety

Three adverse events that were possibly related to vaccination were notable. They included grade 3 asymptomatic neutropenia with onset 27 days after the third vaccination (4-mg group), grade 3 urticaria with onset 4 days after the third vaccination (4-mg group), and grade 2

maculopapular rash with onset 27 days after the second vaccination (8-mg group); the last subject did not receive the third vaccination. All resolved without sequelae and had alternative explanations. Vaccinations were well tolerated, and there were no episodes of severe reactogenicity (table 1). The placebo group reported an incidence of pain similar to that of the vaccine groups but reported less induration and erythema; there was no suggestion of a dose effect.

Fourteen (40% [95% CI, 24%–58%]) of the 35 subjects who received 4- or 8-mg injections had vaccine-induced positive diagnostic ELISA results, and 7 (20% [95% CI, 8%–37%]) had indeterminate Western blot results (table 2). Most commonly, the first positive ELISA result occurred at week 12, with a return to background levels by week 52; there were 6 subjects with a positive ELISA result at week 52.

Vaccine-specific antibody responses

Env-specific antibody responses were observed by IP-Western blotting in postimmunization serum only (figure 2*A*). EnvA-, EnvB-, and EnvC-specific antibody responses were observed (figure 2*B*). The frequency of antibody responses increased with the dose; there were none observed at the 2-mg dose, 8 (40%) of 20 at the 4-mg dose, and 12 (80%) of 15 at the 8-mg dose (figure 2*C*, *black bars*).

A dose effect for antibody response was also suggested by the results of an end-point titration ELISA for EnvA, EnvB, and EnvC (figure 2*C* and 2*D*). A positive result for at least 1 Env protein at week 12 was observed in 24 (60% [95% CI, 43%–75%]) of 40 subjects. All 24 positive subjects had detectable antibody against EnvC, 12 had detectable antibody against EnvA, and 9 had detectable antibody against EnvB. At week 12, 1 (20%) of 5 recipients of 2 mg, 12 (60%) of 20 recipients of 4 mg, and 11 (73%) of 15 recipients of 8 mg had a positive response (figure 2*C*, *hatched bars*). The peak antibody titer for each of the envelope antigens occurred between weeks 8 and 24 and was greatest for EnvC in the 8-mg group (figure 2*D*). Vaccine-induced HIV antibody was observed in only 1 subject by 1 test method in the 2-mg group. Serum neutralizing activity against the MN strain of HIV-1 was <80% in all subjects, indicating that no substantial neutralizing activity was present.

Vaccine-induced T cell responses

T cell responses were detected by both ELISpot and ICS assay and were most frequent to Env antigens. The ICS cytokine gating strategy is shown by a representative analysis at week 12, which demonstrates responses to EnvA, EnvB, and EnvC peptide pools in $CD4^+$ and $CD8^+$ T cells (figure 3). EnvA peptides elicited responses of the greatest magnitude (figure 4*A* and 4*B*). Responses were detected after 2 immunizations in most vaccine recipients, with the highest response rate observed at week 12 (4 weeks after the third immunization) (figure 5 and table 3). The median magnitude of the T cell response as measured by both ELISpot and ICS assay generally peaked at week 10 or 12 for all peptide pools and persisted through week 52 (figure 4*A*). For EnvA, week 12 T cell responses ranged from 0.024% to 0.41% of total $CD4^+$ T cells (as measured by ICS assay), from 0.045% to 0.65% of total $CD8^+$ T cells (as measured by ICS assay), and from 50 to 710 sfc/10^6 PBMCs (as measured by ELISpot assay) (figure 4*B*). False-positive results occurred, with a magnitude just over the threshold, at a frequency of 1 of 48 for the ICS assay for $CD4^+$ T cells, 0 of 48 for the ICS assay for $CD8^+$ T cells, and 2 of 48 for the ELISpot assay (figure 5).

As measured by ELISpot assay, there was a statistically significant difference in the number of positive EnvA, EnvB, and EnvC responses in vaccine recipients ($n = 39$) at week 12, compared with that at baseline ($P<.0001$, $P<.0001$, and $P = .001$, respectively, exact McNemar

tests). Gag, Pol, and Nef responses among vaccine recipients were not significantly different between baseline and week 12.

Positive $CD4^+$ T cell responses as measured by ICS assay for at least 1 of the vaccine-specific antigens were detected in 39 (97.5%) of the 40 vaccine recipients for at least 1 time point by week 12. At week 12, compared with that at baseline, there was a statistically significant difference ($P \leq .0001$ for all, exact McNemar tests) in the number of vaccine recipients with a $CD4^+$ T cell response to EnvA, EnvB, and EnvC but not to Gag, Pol, or Nef.

Positive $CD8^+$ T cell responses as measured by ICS assay for peptides representing at least 1 of the vaccine antigens were detected in 16 (40%) of the 40 vaccine recipients by week 12. At week 12, compared with that at baseline, there was a statistically significant difference ($P = .002$ and $P = .0313$, respectively, exact McNemar tests) in the number of vaccine recipients with a $CD8^+$ T cell response to EnvA and EnvB but not to EnvC; no vaccine recipients had a $CD8^+$ T cell response to Gag, Pol, or Nef.

Although there was a trend toward a greater magnitude (figure 4) and frequency (figure 5) of T cell responses in recipients of the 4- or 8-mg dose than in the recipients of the 2-mg dose, this difference was not statistically significant at week 12. The pattern of responses as measured by ELISpot assay throughout the study was consistent with the pattern as measured by ICS assay. The frequency and magnitude were greatest for EnvA and EnvB, with weaker responses to EnvC, few responses to Gag, and no responses to Nef or Pol (figure 5).

Once T cell responses were detected, they remained detectable in the majority of subjects for the 52 weeks of follow-up (figures 4*A* and 5 and table 3). The functional properties of the vaccine-induced T cell response changed qualitatively over time. A representative T cell cytokine-production profile for IL-2 and IFN-γ at week 12 is shown in figure 6*A*. The $CD4^+$ and $CD8^+$ T cell subsets contain cells that make IL-2 only, IFN-γ only, or both. At week 12, $CD4^+$ T cells tended to make IL-2 only or in combination with IFN-γ, whereas the great majority of $CD8^+$ T cells typically produced IFN-γ only (figure 6*A*). At week 52, both T cell subsets tended to have a higher frequency of IL-2–producing cells and a lower frequency of IFN-γ–producing cells (figure 6*B*).

DISCUSSION

This 4-plasmid HIV-1 DNA candidate vaccine was assessed as safe and well tolerated in healthy uninfected adults. Importantly, DNA immunization induced both HIV-1–specific antibody and T cell responses to the Env proteins of the 3 major subtypes of HIV-1. We have shown that DNA as a gene-delivery platform for HIV-1 vaccine antigen expression can induce significant immune responses that are sustained for at least 1 year in the majority of antigen-naive subjects. The concept of using bacteria-derived plasmid DNA to deliver vaccine antigens has many attractive features, including ease and flexibility of construction, scalable manufacturing capacity, stability, intracellular production of vaccine antigen, transient expression, no induction of antivector immunity, induction of a balanced $CD4^+$ and $CD8^+$ T cell response as well as of antibody, and lack of significant local or systemic reactogenicity. The broad immunogenicity of the VRC HIV-1 DNA candidate vaccine probably reflects a combination of factors, including optimization of vector design, manufacturing methods, delivery, sample processing, and immunological end-point measurements.

A notable feature of the immunogenicity analysis was the response to Env antigens relative to Gag, Pol, and Nef. The dominant antigenicity of Env was observed for both antibody and T cell responses, with EnvA peptides eliciting the greatest T cell responses and EnvC binding the most vaccine-induced antibody. All the genes encoding the vaccine antigens were codon modified to improve expression. The Env constructs received additional modification to

remove the cleavage site, the fusion peptide of gp41, a region of the interspace between the 2 gp41 heptad repeats, and the cytoplasmic domain [3], but there is no indication that these changes were responsible for liberating additional T cell epitopes. The *gag/pol/nef* construct was designed to remove the frame shift, resulting in improved expression of Pol and Nef, at the expense of 66 aa from the carboxy-terminus of Gag [18]. This construct was immunogenic in preclinical testing and exhibited strong protein expression in vitro [4]. Here, the Gag, Pol, and Nef antigens elicited only a modest induction of $CD4^+$ T cell responses despite representing 50% of the plasmid mixture by weight. It is not known why the induction of Gag-, Pol-, and Nef-specific responses were lower than Env-specific responses, but there are several possible explanations for increased Env immunogenicity. First, since Env is a membrane glycoprotein, its intracellular trafficking and mode of antigen presentation differs from those of the internal viral proteins. Second, Gag, Pol, and Nef are not glycosylated, are synthesized in the cytoplasm, and localize to a subcellular compartment different from that of Env. Therefore, it is possible that the steps involved in antigen processing and the kinetics of antigen presentation may differ. Finally, our experiments in animal models indicate that Env contains more $CD4^+$ T cell epitopes than does Gag or other proteins [19]. Thus, the increased immunogenicity of Env is potentially an intrinsic property of the expressed antigen.

Antibody responses induced by this 4-plasmid combination vaccine were detected by IP-Western blotting and research ELISA for vaccine-specific antigens as well as by commercial ELISA and Western blotting for native viral proteins (table 2). Analysis of neutralization did not reveal significant activity. On the basis of animal studies, high levels of binding antibody and neutralizing activity are not anticipated after DNA immunization alone but can be achieved after subsequent boosting with replication-defective recombinant adenovirus vectors [20]. The induction of measurable antibody to clades A, B, and C Env proteins with DNA alone in humans is encouraging and suggests that DNA vaccination is a viable strategy for priming both cellular and humoral responses. Whether the response to all the Env proteins indicates cross-reactivity between clades or between clade-specific antibodies is not known. As multivalent vaccines become more immunogenic and clinical evaluation expands, the complexity of interpreting routine commercial diagnostic tests will increase. Alternative approaches to serological diagnosis, such as gene-based diagnostic approaches, will be needed in the future.

This DNA vaccine consistently induced T cell responses. Envspecific $CD4^+$ T cell responses were present in nearly all subjects. Detection of this response may be attributed to the development of a highly sensitive flow cytometry–based assay that detects IFN-γ and/or IL-2 on the same channel. Other studies have shown that most of the $CD4^+$ T cell response can be captured by the combination of these 2 cytokines [21,22]; if IFN-γ alone is used as the end point, a portion of the $CD4^+$ T cell response is missed. $CD8^+$ T cells are key effectors for clearing virus-infected cells [23] and have been associated with control of lentivirus replication in both nonhuman primates (NHPs) and humans [24–32]. Twelve (34%) of the 35 vaccine recipients who received the 4-mg or 8-mg doses had detectable HIV Env–specific $CD8^+$ T cell responses at week 12. The magnitude of positive responses ranged from just above background to 0.65% of total $CD8^+$ T cells. When detected, the magnitude of the $CD8^+$ T cell response is comparable to that seen in NHP studies of similar vaccines, although typically $CD8^+$ T cell responses can be detected in 100% of the animals [33,34]. An interesting feature of the T cell response was that, over the course of a year, a higher frequency of EnvA-specific $CD4^+$ and $CD8^+$ T cells made IL-2 only. Reports that show that IFN-γ– producing $CD4^+$ T cells are not long lived [35] and that IL-2– producing $CD8^+$ T cells have a greater proliferative capacity in HIV-1–infected patients with nonprogressive disease [36] suggest that these IL-2–producing T cells may have the capacity for expansion after subsequent antigen exposure.

Demonstrating the safety and immunogenicity of a multigene, multiclade HIV DNA vaccine is an important step in the development of a globally relevant vaccination strategy. The capacity

to make precise measurements of vaccine-induced T cell responses will improve the chances of defining correlates of immune protection in future trials.

VACCINE RESEARCH CENTER (VRC) 004 STUDY TEAM

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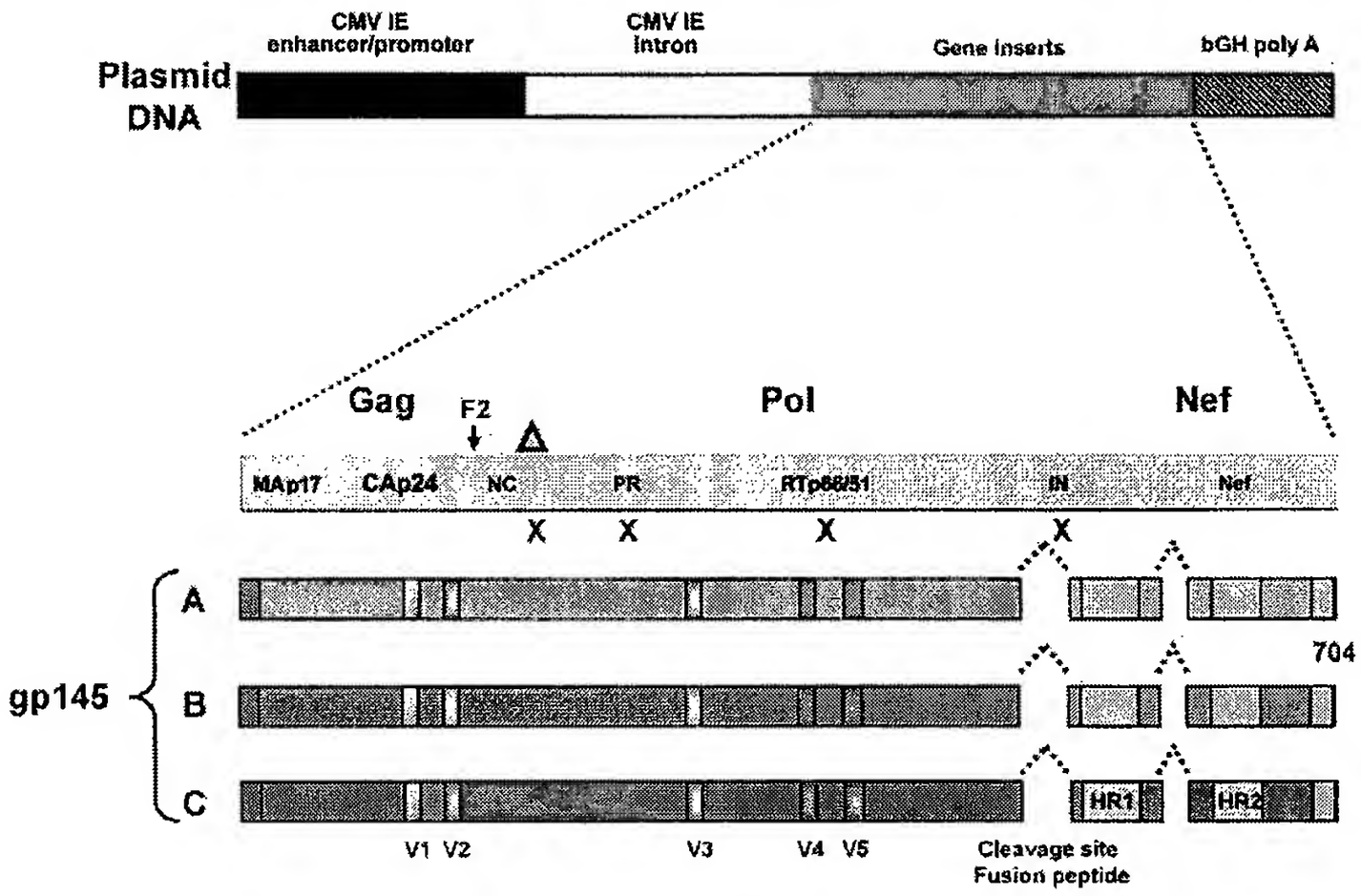

Figure 1.

Schematic of the DNA vaccine design. Four separate DNA plasmids were produced by inserting individual HIV-1 gene constructs into the pVR1012 backbone under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter, followed by the bovine growth hormone polyadenylation (bGH poly A) sequence [3,12]. The synthetic *gag* gene is from the clade B strain HXB2 (GenBank accession no. K03455), the synthetic *pol* gene *(pol/h)* is from the clade B strain NL4–3 (GenBank accession no. M19921), and the synthetic *nef* gene *(nef/h)* is from the clade B strain PV22 (GenBank accession no. M19921). Mutations (indicated by Xs), including the deletion of the carboxy-terminus of Gag (indicated by the triangle), were introduced in the protease and reverse-transcriptase genes to prevent processing of the *pol* gene products and to reduce the potential for functional enzymatic activity. This resulted in a fusion protein that directly reads through the frame shift in Gag (F2) through Pol and into Nef. This gene product is not able to assemble or produce pseudoparticles. To create synthetic gp145, versions of the envelope genes were truncated immediately downstream of the transmembrane domain of gp41. In each construct, the cleavage site and fusion peptide at the junction of gp120 and gp41 were deleted, and a portion of the interspace between the 2 heptad-repeat regions in gp41 was deleted. The Env gene products are primarily cell associated rather than secreted. The EnvA sequence is from 92rw020 (CCR5 tropic; GenBank accession no. U08794), the EnvB sequence is from HXB2 (CXCR4 tropic; GenBank accession no. K03455), and the EnvC sequence is from 97ZA012 (CCR5 tropic; GenBank accession no. AF286227). HR1–HR2, heptad-repeat regions in gp41; IN, integrase; NC, nucleocapsid; PR, protease; V1–V5, variable regions in envelope.

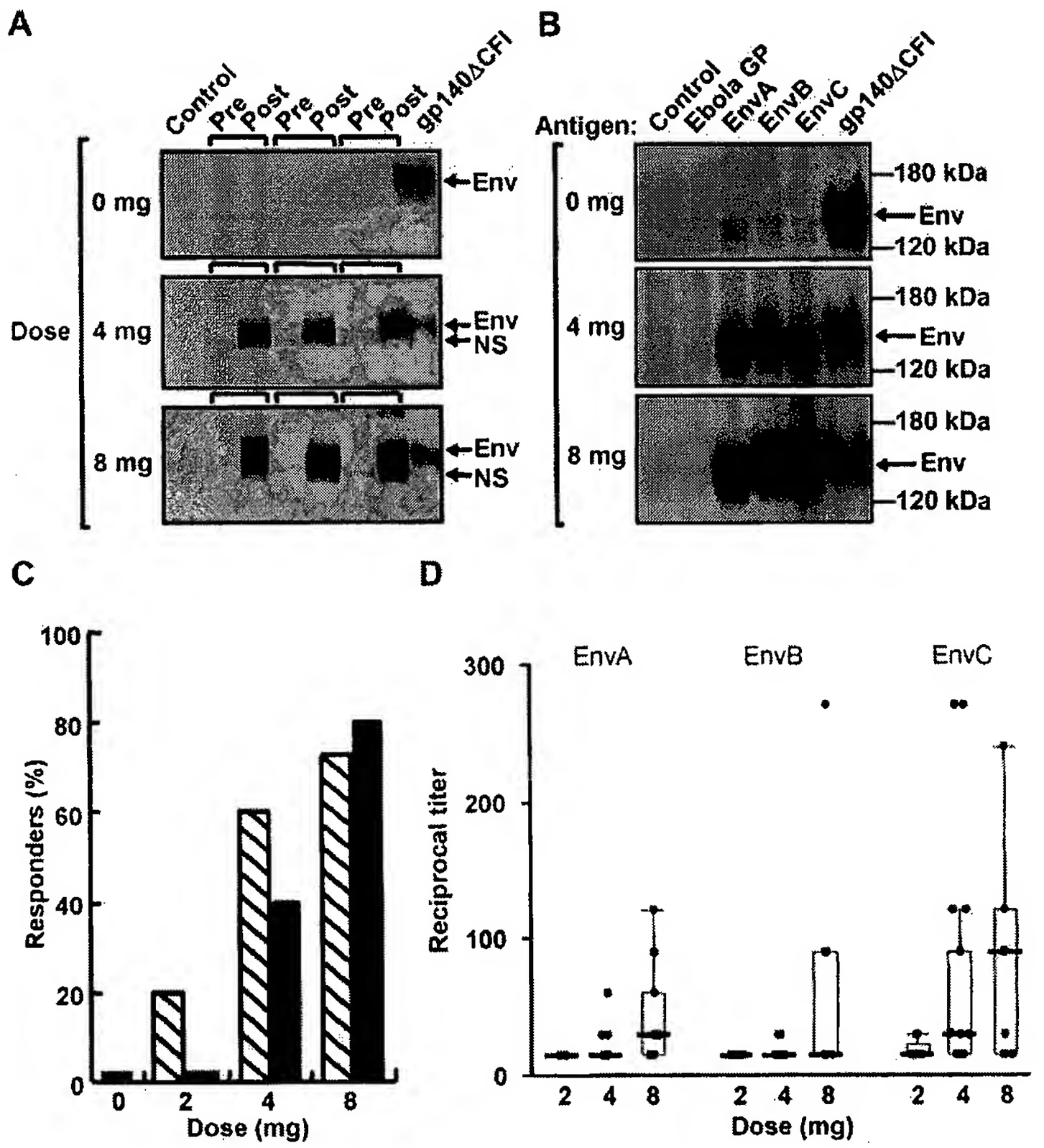

Figure 2.

Induction, specificity, and dose response of the vaccine-induced antibody response to clades A, B, and C Env proteins. *A,* Western blot analysis of Env antigens captured by immunoprecipitation using prevaccination (pre) and 12-week (post) serum samples from representative placebo recipients (0 mg) and vaccine recipients (4- or 8-mg doses). Arrows indicate the EnvB-specific band distinguished from a small nonspecific (NS) background band. *B,* Specific reactivity of antibody induced by vaccination to EnvA, EnvB, and EnvC but not to an unrelated Ebola virus glycoprotein–negative control (Ebola GP). All proteins were produced in the supernatants of 293T cells transfected with the vaccine plasmids. Arrows indicate the Env-specific band. Results are shown for representative subjects from the placebo group (0 mg) and from the 4- and 8-mg groups. No positive bands were detected for any of the placebo recipients at any time point in the study. *C,* Frequency of positive antibody responders to purified proteins at week 12 as measured by end-point titration ELISA *(hatched bars)* or by immunoprecipitation followed by Western blotting *(black bars),* as a function of dose. No antibody was detected by these methods for placebo recipients or for vaccine recipients before immunization. *D,* Peak response to purified proteins for EnvA, EnvB, and EnvC as measured by end-point titration ELISA for each subject, by dose group. The peak response (reciprocal

titer) occurred between weeks 8 and 24. The box plots indicate the median, 25th, and 75th percentiles for each dose level, and the error bars show the 5th and 95th percentile.

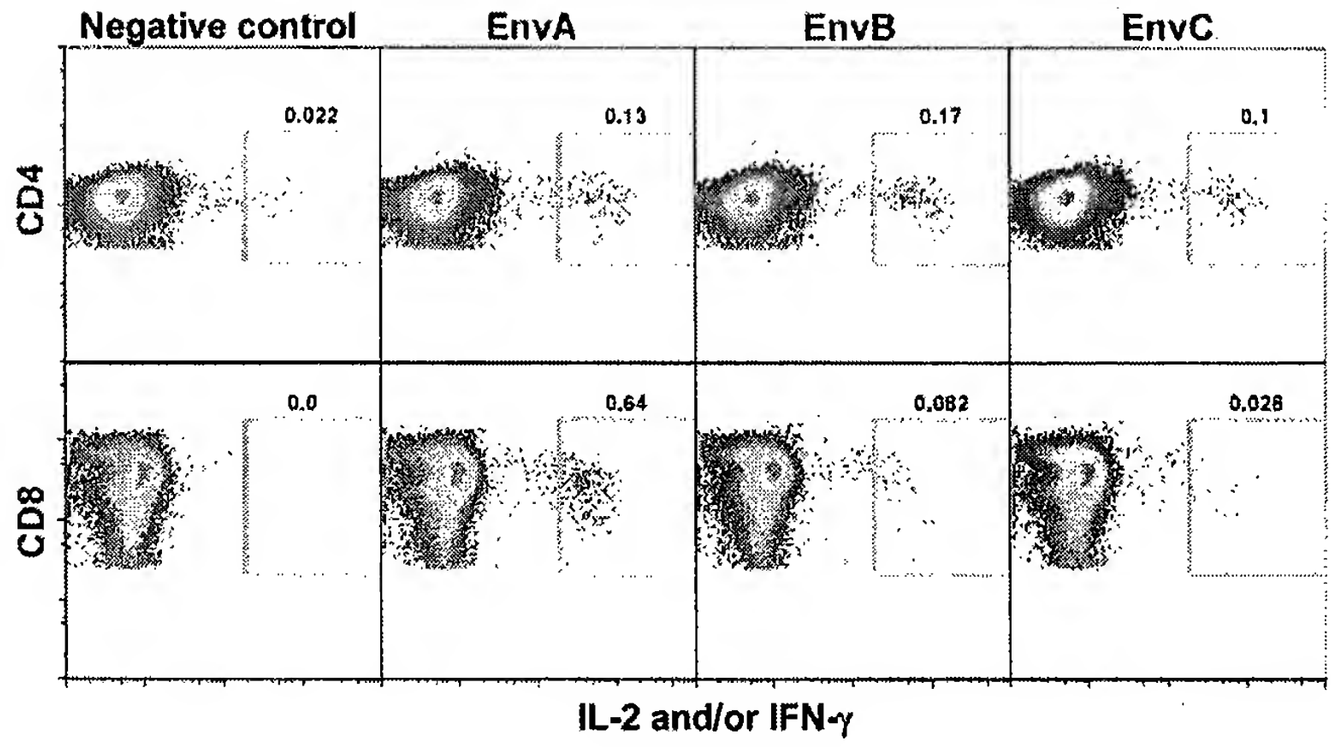

Figure 3.

Measurement of HIV-specific T cell responses. The T cell response measurements were largely based on flow-cytometric detection of intracellular cytokine production in peripheral-blood mononuclear cells (PBMCs) stimulated with peptide pools representing the vaccine antigen. Because this is a relatively new approach for determining immunogenicity in clinical trials of preventive vaccines, an example of the primary data and gating strategy for this method is demonstrated for a representative subject. $CD4^+$ and $CD8^+$ T cell responses are shown for a single recipient of the 4-mg dose at week 12 (4 weeks after completion of the vaccination schedule). PBMCs were incubated either with costimulatory antibodies only (negative control) or with EnvA, EnvB, or EnvC peptide pools, and production of interferon (IFN)–γ and/or interleukin (IL)–2 was measured on the same wavelength. Gating *(pink box)* reflects cells producing higher levels of cytokine. The numeric value above each gate represents the percentage of total $CD4^+$ or $CD8^+$ T cells producing cytokine.

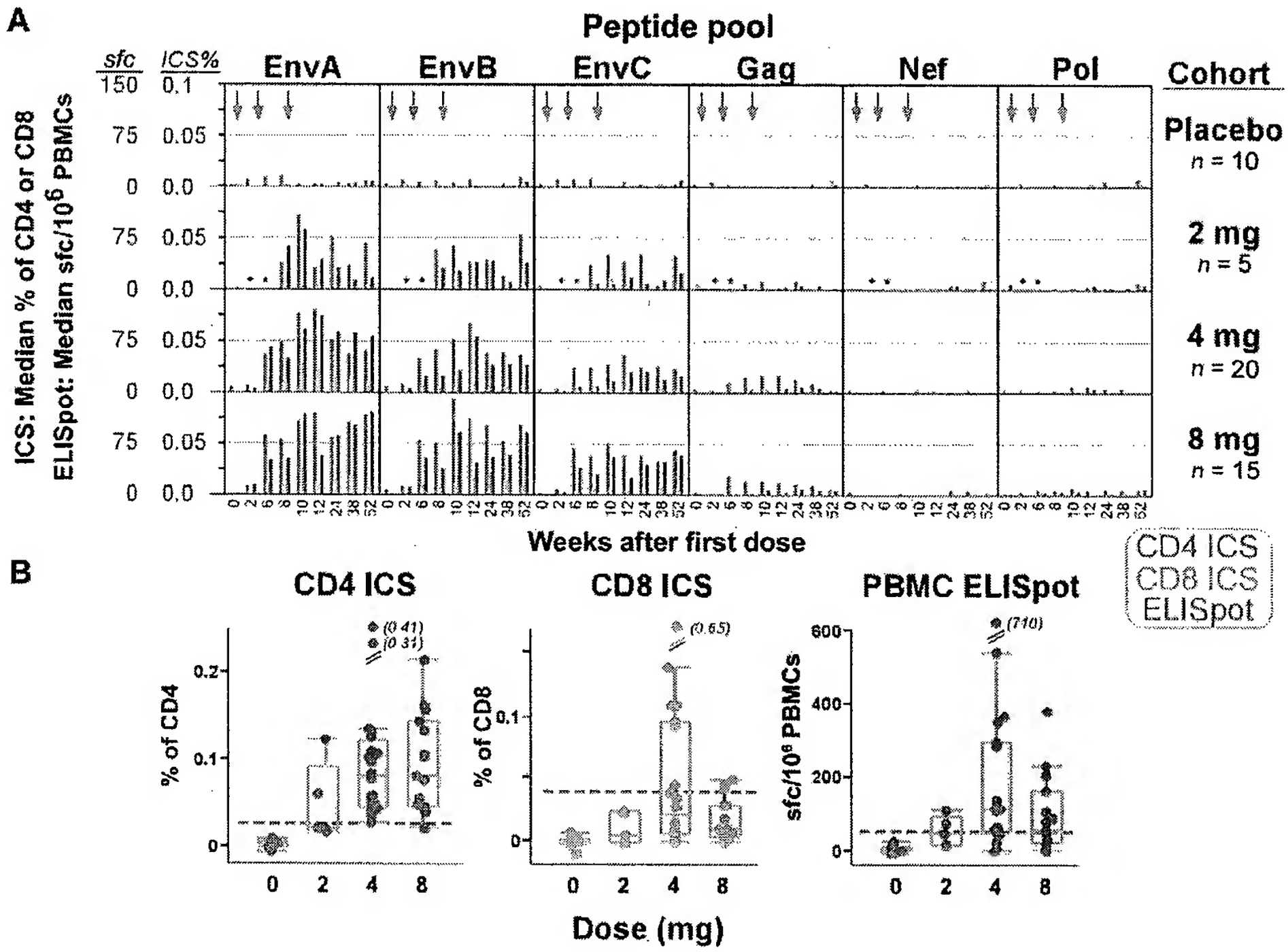

Figure 4.

Magnitude of T cell responses to specific vaccine components. T cell responses were measured by intracellular cytokine staining (ICS) assay to detect interferon (IFN)–γ and/or interleukin-2 and by IFN-γ enzyme-linked immunospot (ELISpot) assay for all placebo and vaccine recipients. *A,* Median magnitudes of peptide pool–specific responses, shown as a percentage of total $CD4^+$ or $CD8^+$ T cells for the ICS assay (scale 0–0.1) and as the no. of spot-forming cells per 10^6 peripheral-blood mononuclear cells (PBMCs) for the ELISpot assay (scale 0 –150), for all subjects, by dose group. The time course for each subject is shown by study week on the *X*-axis; arrows along the top of the graph designate time points for immunizations (study weeks 0, 4, and 8). Within each of the 24 boxes, the entire time course of the study is represented. Each column shows the responses to a peptide pool representing the respective vaccine antigens (EnvA, EnvB, EnvC, Gag, Nef, and Pol). Each row represents a dose cohort (placebo recipients, 2-mg recipients, 4-mg recipients, and 8-mg recipients). Red bars represent $CD4^+$ T cell responses as measured by ICS assay, green bars represent $CD8^+$ T cell responses as measured by ICS assay, and blue bars represent $CD4^+$ or $CD8^+$ T cell responses as measured by ELISpot assay. Time points without samples for analysis are represented by asterisks. *B,* Magnitudes of specific T cell responses to a peptide pool for EnvA at study week 12, by dose group. EnvA-specific responses are shown for each subject as measured by 3 assays; shown are the percentages of $CD4^+$ or $CD8^+$ T cells producing cytokine as measured by ICS assay and the no. of spot-forming cells per 10^6 PBMCs as measured by ELISpot assay. The left plot shows EnvA-specific $CD4^+$ T cell responses, the middle plot shows EnvA-specific $CD8^+$ T cell responses, and the right plot shows EnvA-specific $CD4^+$ or $CD8^+$ responses. The box plots

indicate the median, 25th, and 75th percentiles for each dose level, and the error bars show the 5th and 95th percentiles. The horizontal dashed line on each plot indicates the laboratory threshold of positivity for each assay (for the ICS assay, 0.0241% for $CD4^+$ T cells and 0.0445% for $CD8^+$ T cells; for the ELISpot assay, 50 sfc/10^6 PBMCs).

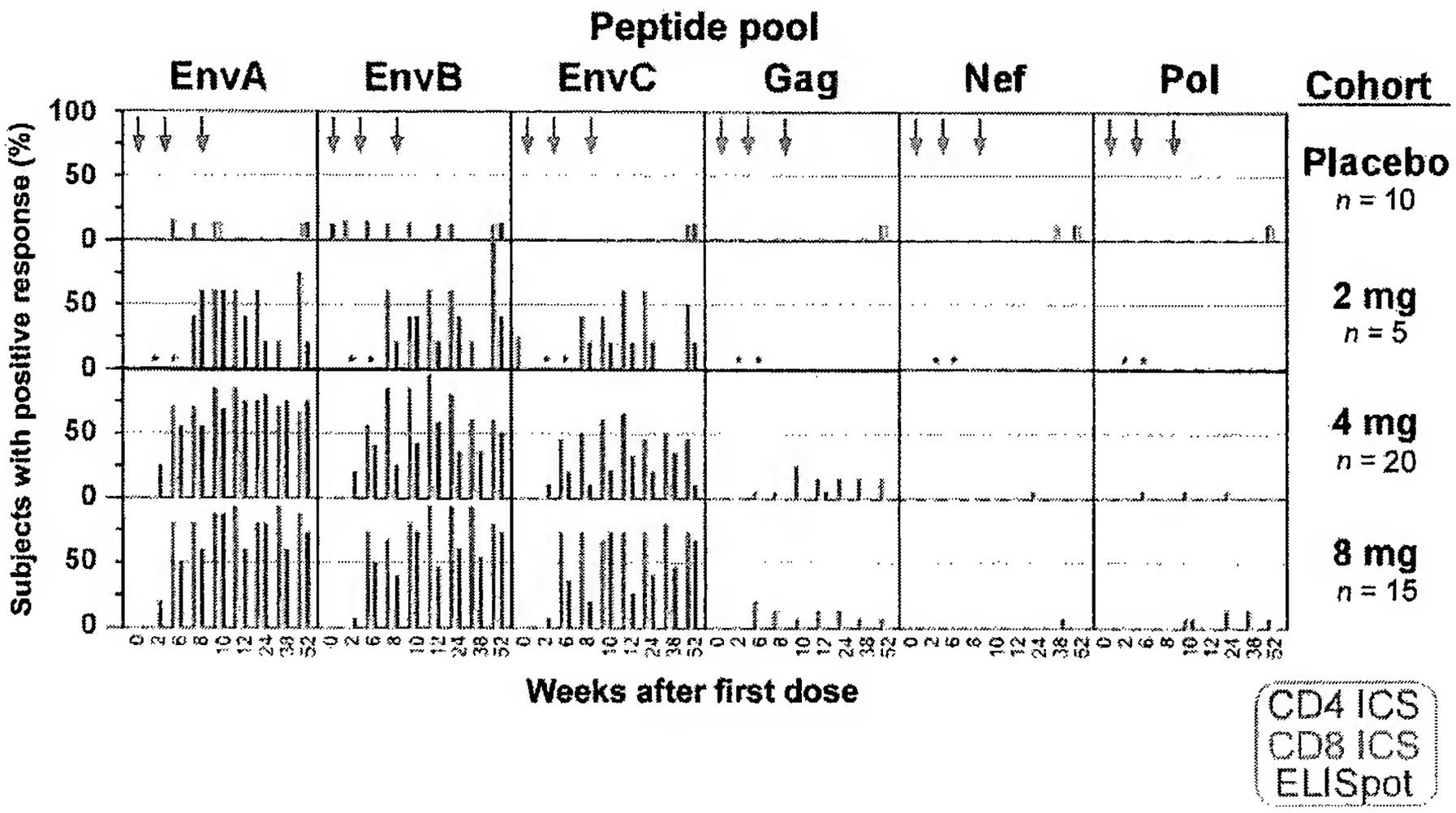

Figure 5.

Frequencies of subjects with detectable T cell responses. All T cell responses for each antigen are shown for all subjects at all time points. Each of the 24 boxes shows the frequency of response to a different antigen in a different dose group for the entire time course of the study. The *Y*-axis of each of the 24 boxes shows the frequency of response as the percentage of subjects in a dose group with a positive response to the respective peptide pool for each assay. The *X*-axis shows the time course of the study by study week (0–52) in each of the 24 plots. Each row represents a dose cohort (placebo recipients, 2-mg recipients, 4-mg recipients, and 8-mg recipients). Each column represents the peptide pool used for stimulation of peripheral-blood mononuclear cells (EnvA, EnvB, EnvC, Gag, Nef, and Pol). Red bars represent $CD4^+$ T cell responses as measured by intracellular cytokine staining (ICS) assay, green bars represent $CD8^+$ T cell responses as measured by ICS assay, and blue bars represent $CD4^+$ or $CD8^+$ T cell responses as measured by enzyme-linked immunospot (ELISpot) assay. No week 2 data and only 1 sample from week 6 are included for the 2-mg cohort (time points without samples for analysis are represented by asterisks).

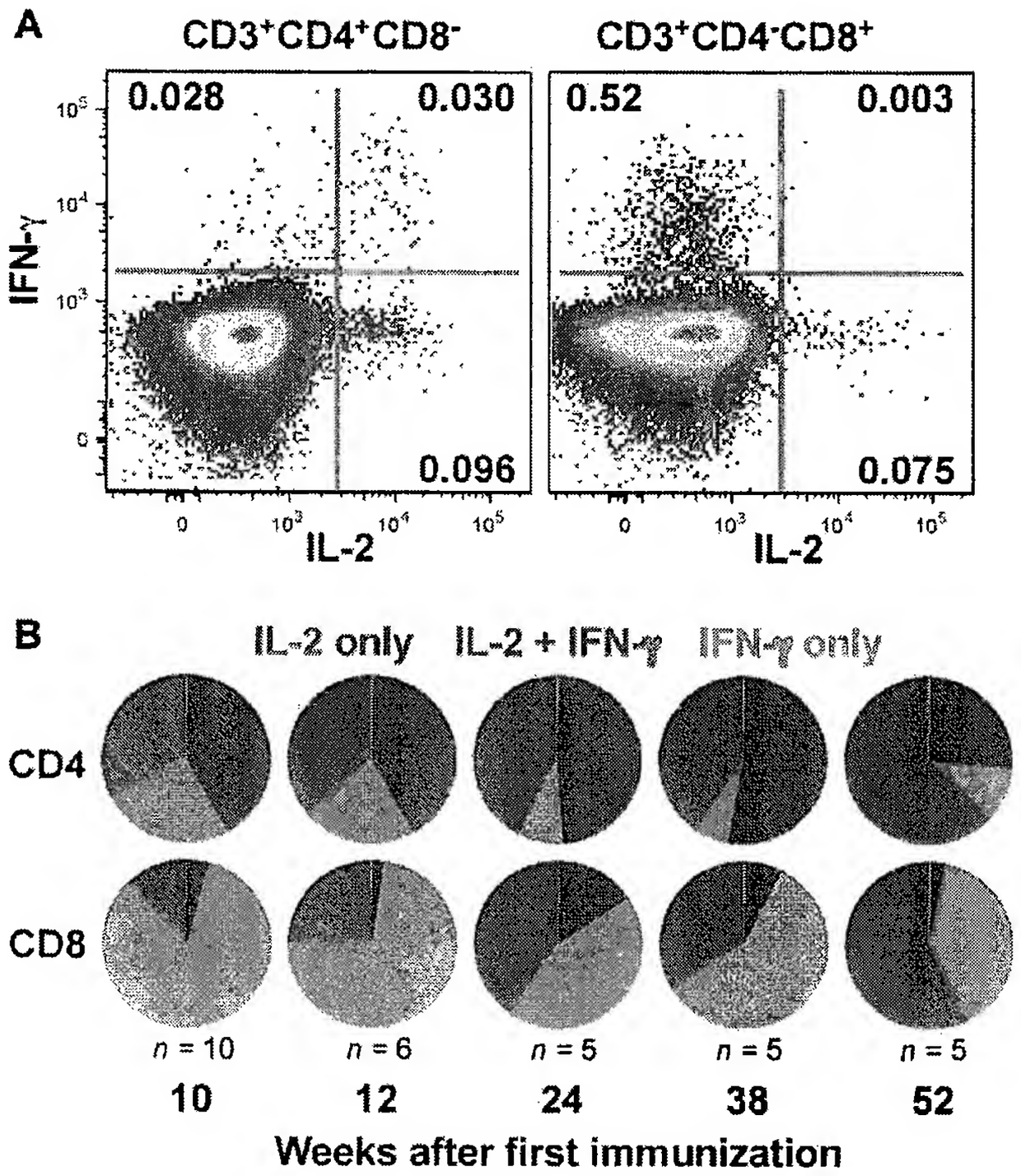

Figure 6.

Evolution of cytokine-expression patterns in vaccine-induced T cells over time. Multiparameter flow cytometry was performed in a subset of subjects to define the relative frequency of interferon (IFN)–γ and interleukin (IL)–2 production in T cells stimulated by the vaccine-specific peptide pools. *A,* Response of either $CD4^+$ or $CD8^+$ T cells from a representative vaccine recipient is shown at study week 12 after stimulation with the EnvA peptide pool. For each of the 2 plots, the *Y*-axis shows $CD3^+$ ($CD4^+$ or $CD8^+$) T cells producing IFN-γ, whereas the *X*-axis shows IL-2 production. The plot on the left demonstrates $CD4^+$ T cell production of IL-2 alone (in the bottom right quadrant; 0.096%), IFN-γ alone (in the top left quadrant; 0.028%), or both (in the top right quadrant; 0.030%). The plot on the right demonstrates $CD8^+$ T cell production of IFN-γ alone (in the top left quadrant; 0.52%), IL-2 alone (in the bottom right quadrant; 0.075%), or both (in the top right quadrant; 0.003%). Over time, the relative frequency of cells producing IFN-γ appeared to diminish, and the relative frequency of vaccine-specific IL-2–producing cells appeared to increase, as shown in panel B by use of pie charts demonstrating the average cytokine-expression pattern from a subset of vaccine recipients with the highest initial responses to the EnvA peptide pool over the 52-week time course of the study. There are 2 sets of pie charts: the top row shows $CD4^+$ T cells, and the bottom row shows $CD8^+$ T cells, both as measured by intracellular cytokine staining assay. Five time points of the study are represented (week 10, 12, 24, 38, and 52), and the no. of representative subjects included at each time point is listed. The pie charts show the distribution of cytokine-expression patterns of each T cell responding to the EnvA peptide pool: the blue

segments represent T cells producing IL-2 only, the red segments represent T cells producing both IL-2 and IFN-γ, and the green segments represent T cells producing IFN-γ only.

Table 1

Subject demographics and vaccine reactogenicity.

Category, parameter	Placebo recipients (n = 10)	Vaccine recipients (n = 40)
Demographics		
Sex		
Male	8 (80)	22 (55)
Female	2 (20)	18 (45)
Age		
18–20 years	1 (10)	2 (5)
21–30 years	4 (40)	22 (55)
31–40 years	5 (50)	16 (40)
Mean ± SD, years	30 ± 7	29 ± 5
Range, years	20–39	18–38
Race		
White	7 (70)	34 (85)
Black or African American	1 (10)	4 (10)
Asian/Pacific Islander	2 (20)	1 (2.5)
American Indian/Alaskan Native	0 (0)	0 (0)
Multiracial	0 (0)	1 (2.5)
Ethnicity		
Non-Hispanic/Latino	9 (90)	39 (98)
Hispanic/Latino	1 (10)	1 (2.5)
Reactogenicity summary[a]		
Local		
None	2 (20)	1 (2.5)
Mild	7 (70)	30 (75)
Moderate	1 (10)	9 (22.5)
Severe	0 (0)	0 (0)
Systemic		
None	3 (30)	10 (25)
Mild	5 (50)	22 (55)
Moderate	2 (20)	8 (20)
Severe	0 (0)	0 (0)

NOTE. Data are no. (%) of subjects, unless otherwise indicated.

[a] Each subject was counted once for his or her worst severity score during the 7 days after each vaccination.

Table 2

Frequency of vaccine-induced antibody responses.

Group	Commercial ELISA	Commercial Western blot	ELISA EnvC	IP—Western blot
2 mg ($n = 5$)	0 (0)	0 (0)	1 (20)	0 (0)
4 mg ($n = 20$)	11 (55)	5 (25)	12 (60)	8 (40)
8 mg ($n = 15$)	3 (20)	2 (13)	11 (73)	12 (80)
Placebo[a] ($n = 8$)	0 (0)	0 (0)	0 (0)	0 (0)

NOTE. Data are no. (%) of subjects. IP, immunoprecipitation.

[a]Two subjects who became infected with HIV, both in the placebo group, are not included in this table. All other subjects ($n = 48$) remained polymerase chain reaction (PCR) negative throughout the study. Aside from the results for the 2 HIV-infected subjects, the Western blot results were all in the indeterminate category. A positive Western blot result required a band at p24 in addition to a band for at least 1 of the envelope glycoproteins (gp41, gp120, or gp160). If there were bands present that did not meet the positivity criteria, the result was reported as indeterminate.

Table 3

Frequency of T cell responses at weeks 12 and 52, as assessed by intracellular cytokine staining assay.

T cell subset, peptide pool	Week 12 frequency		Week 52 frequency	
	4-mg group (n = 20)	8-mg group (n = 15)	4-mg group (n = 20)	8-mg group (n = 15)
$CD4^+$ T cells				
EnvA	17 (85)	14 (93)	13 (65)	13 (87)
EnvB	19 (95)	14 (93)	12 (60)	12 (80)
EnvC	13 (65)	11 (73)	9 (45)	11 (73)
Gag	3 (15)	2 (13)	3 (15)	1 (7)
Nef	0 (0)	0 (0)	0 (0)	0 (0)
Pol-1[a]	0 (0)	0 (0)	0 (0)	1 (7)
Pol-2[a]	0 (0)	0 (0)	1 (5)	2 (13)
Any	20 (100)	14 (93)	14 (70)	14 (93)
$CD8^+$ T cells				
EnvA	7 (35)	3 (20)	4 (20)	1 (7)
EnvB	2 (10)	3 (20)	1 (5)	1 (7)
EnvC	0 (0)	1 (7)	0 (0)	0 (0)
Gag	0 (0)	0 (0)	0 (0)	0 (0)
Nef	0 (0)	0 (0)	0 (0)	0 (0)
Pol-1[a]	0 (0)	0 (0)	0 (0)	0 (0)
Pol-2[a]	0 (0)	0 (0)	0 (0)	0 (0)
Any	7 (35)	5 (33)	4 (20)	1 (7)

NOTE. Data are no. (%) of subjects.

[a]Pol protein is too large to include all peptides in 1 pool; therefore, the Pol peptides were divided into 2 pools.

EXHIBIT

AO

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A DNA Vaccine for Ebola Virus Is Safe and Immunogenic in a Phase I Clinical Trial▽†

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Ebola viruses represent a class of filoviruses that causes severe hemorrhagic fever with high mortality. Recognized first in 1976 in the Democratic Republic of Congo, outbreaks continue to occur in equatorial Africa. A safe and effective Ebola virus vaccine is needed because of its continued emergence and its potential for use for biodefense. We report the safety and immunogenicity of an Ebola virus vaccine in its first phase I human study. A three-plasmid DNA vaccine encoding the envelope glycoproteins (GP) from the Zaire and Sudan/Gulu species as well as the nucleoprotein was evaluated in a randomized, placebo-controlled, double-blinded, dose escalation study. Healthy adults, ages 18 to 44 years, were randomized to receive three injections of vaccine at 2 mg ($n = 5$), 4 mg ($n = 8$), or 8 mg ($n = 8$) or placebo ($n = 6$). Immunogenicity was assessed by enzyme-linked immunosorbent assay (ELISA), immunoprecipitation-Western blotting, intracellular cytokine staining (ICS), and enzyme-linked immunospot assay. The vaccine was well-tolerated, with no significant adverse events or coagulation abnormalities. Specific antibody responses to at least one of the three antigens encoded by the vaccine as assessed by ELISA and $CD4^+$ T-cell GP-specific responses as assessed by ICS were detected in 20/20 vaccinees. $CD8^+$ T-cell GP-specific responses were detected by ICS assay in 6/20 vaccinees. This Ebola virus DNA vaccine was safe and immunogenic in humans. Further assessment of the DNA platform alone and in combination with replication-defective adenoviral vector vaccines, in concert with challenge and immune data from nonhuman primates, will facilitate evaluation and potential licensure of an Ebola virus vaccine under the Animal Rule.

Outbreaks of infection with Ebola virus result in a rapid and severe disease with high mortality, for which there is currently no licensed antiviral treatment or vaccine. While outbreaks remain unpredictable, they have occurred with increasing frequency in equatorial Africa, west of the Rift Valley, where they have infected both humans and nonhuman primates and have significantly depleted chimpanzee and gorilla populations in Central Africa. Although a potential reservoir has been suggested (15), the risk of zoonotic transmission remains high and unpredictable (1). Announcements of Ebola virus outbreaks cause widespread fear and have socioeconomic consequences beyond the direct impact on infected persons. Outbreaks of Ebola virus infection have become more frequent since its discovery in 1976 and reemergence in 1995, and there are now areas in which the infection appears to be endemic. Although there have been no human outbreaks of Ebola virus in the United States, the virus caused an outbreak in imported laboratory nonhuman primates in Reston, Va., in 1989. It is also considered to be a potential bioweapon. For these reasons, vaccine development for Ebola virus and other filoviruses has become a priority.

Outbreaks of hemorrhagic fever caused by the Ebola virus are associated with high mortality rates. The highest lethality is associated with the Zaire subtype, one of four species identified to date (9, 21). An outbreak of hemorrhagic fever reported in October 2000 in the Gulu district of Uganda was confirmed as Ebola virus and resulted in the deaths of dozens of people (6). Another outbreak in Gabon and the Republic of Congo likely involved several independent introductions. It continued from 2001 through 2003 and resulted in more than 100 deaths (3, 28). The triggers for such outbreaks are not understood, although there may be a correlation with climatic changes (31). These periodic but devastating outbreaks underscore the difficulty in controlling this virus that emerges periodically via uncertain primary transmission routes and then disappears into an unclearly defined natural reservoir.

Infection with Ebola virus initially results in an influenza-like syndrome that progresses to severe illness manifested by co-

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† Supplemental material for this article may be found at http://cvi.asm.org/.

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agulation abnormalities, disseminated intravascular coagulation, multi-organ system involvement, and an exaggerated but nonprotective inflammatory response (1). Fatality rates range from 50 to 90%, and death is frequently due to bleeding and hypotensive shock (22). The rapid advancement of severe disease following Ebola virus infection allows little opportunity to develop natural immunity, and there is no effective antiviral therapy currently available. Vaccination offers a propitious intervention to prevent infection and limit spread as well as an important public health benefit for health care workers involved in care of patients and the containment of outbreaks.

Because of the potential safety concerns associated with using conventional vaccination strategies, such as attenuated or inactivated Ebola virus as an immunogen, the vaccination strategies that have been evaluated in published preclinical studies to date for Ebola virus have focused on the use of live and replication-defective vectors and virus-like particles. Published studies have included expression of Ebola virus protein subunits in live vaccinia virus vectors, selected DNA plasmids, Ebola virus-like particles, replication-competent vesicular stomatitis virus, Venezuela equine encephalitis virus replicons, recombinant parainfluenza virus type 3, replication-defective recombinant adenovirus (rAd), and DNA combined with rAd prime-boost strategies (5, 12, 14, 19, 22, 24, 26, 31). While many of these approaches have been evaluated in a nonhuman primate model (10), only DNA/rAd-, rAd-, or vesicular stomatitis virus-based vaccines have shown efficacy in primates.

DNA vaccination conferred an Ebola virus-specific immune response in guinea pigs and mice (4, 25, 32) that protected against a challenge with Ebola virus adapted to produce lethal infection in rodents (1, 4, 8). Humoral and T-cell-mediated immunity were elicited in these animal models, but antibody titers appeared to correlate better with protection following immunization with plasmids carrying genes for Ebola virus Zaire proteins. DNA vaccination followed by a boost with recombinant adenoviral vectors encoding Ebola viral proteins uniformly protected nonhuman primates from an otherwise-lethal dose of Ebola virus (25). Protection correlated with the development of Ebola virus-specific $CD8^+$ T-cell and antibody production. The vaccinated animals remained protected and asymptomatic following challenge with a lethal dose of the highly pathogenic, wild-type, 1976 Mayinga strain of Ebola virus Zaire (22, 24, 25).

Ideally, an effective vaccine would provide immunity to the multiple Ebola virus species that have been isolated in human infections and may require multiple antigenic specificities. There is a concern that combining multiple expression vectors in the same vaccine may result in interference of expression of some of the constructs (23). However, a multigene vaccine containing antigens for Ebola virus glycoproteins from the Zaire, Ivory Coast, and Sudan viruses induced specific immune responses to all three subtypes without evidence of interference in an animal model (24, 25). Additionally, a series of studies showed that both GP and sGP (a soluble form of the glycoprotein) conferred optimal protection in the guinea pig model (25, 32). We report the results of the first human clinical trial of a candidate Ebola virus DNA vaccine and show that plasmids expressing Ebola virus GP (Zaire [Z]), GP (Sudan/Gulu [S/G]), and NP (Z) are safe and well-tolerated and induce Ebola virus-specific antibody and T-cell responses in healthy adults.

MATERIALS AND METHODS

Study design. Protocol VRC 204 was a single-site, phase I, randomized, placebo-controlled, double-blinded, dose escalation study to examine safety and tolerability, dose, and immune response to an investigational Ebola virus plasmid DNA vaccine. Healthy adult volunteers 18 to 44 years of age were recruited at the NIAID Vaccine Research Center Clinic, National Institutes of Health (Bethesda, Md.). Human experimental guidelines of the U.S. Department of Health and Human Services were followed in the conduct of clinical research, and the protocol was reviewed and approved by the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board. Three sequential groups of volunteers were enrolled between November 2003 and July 2004 to receive placebo or vaccine at doses of 2.0 mg, 4.0 mg, and 8.0 mg, respectively. Group 1 subjects ($n = 7$) were randomized in a ratio of 5 vaccine/2 placebo; group 2 ($n = 10$) and group 3 ($n = 10$) subjects were randomized in a ratio of 8 vaccine/2 placebo. Total enrollment was 27 volunteers (21 vaccine, 6 placebo). In all groups, the vaccine was administered on study days 0, 28 ± 7, and 56 ± 7 (with at least 21 days between injection days).

Safety through at least 2 weeks after the second injection at each dose level was reviewed by a data and safety monitoring board prior to enrolling volunteers into the next dose level group. For the 2.0-mg and 4.0-mg immunizations, a single dose of vaccine or placebo required a volume of 1.0 ml, administered by intramuscular injection in the lateral deltoid muscle. The maximum concentration of vaccine formulation is 4 mg/ml; therefore, two 1.0-ml injections (one intramuscular injection in each deltoid muscle) of this formulation were necessary to deliver the 8.0-mg dose. Placebo was administered the same way as vaccine for each dosage group. All intramuscular injections were administered with the Biojector 2000 needle-free injection management system. Adverse reactions were evaluated by laboratory and clinical evaluations at scheduled study visits, coded using the *Medical Dictionary for Regulatory Activities*, and severity graded using a scale of 0 to 5. Solicited reactogenicity was collected by study subject report on 7-day diary cards. Subjects were followed for a total of 12 months, and the study was completed in August 2005.

Vaccine. This multigene plasmid DNA vaccine is a mixture of three plasmids in equal concentrations that were constructed to produce three Ebola virus proteins designed to elicit broad immune responses to multiple Ebola virus subtypes. These proteins include the nucleoprotein (NP) derived from the Zaire strain of Ebola virus, which exhibits highly conserved domains, as well as two glycoproteins (GP), which mediate viral entry, from the Zaire strain (homologous to the Ivory Coast strain) and the Sudan/Gulu species (associated with recent outbreaks of hemorrhagic fever in Africa). The Ebola virus GP genes expressed by plasmid DNA constructs in this vaccine contain deletions in the transmembrane region of GP that were intended to eliminate potential cellular toxicity observed in the in vitro experiments using plasmids expressing the full-length wild-type GPs (33). In addition, the Ebola virus GP inserts have been modified to optimize expression in human cells. The three plasmids in this vaccine are incapable of replication in animal cells and would not permit the generation of an infectious virion even if recombination or gene duplication were to occur.

The vaccine plasmids were prepared by cloning the Ebola virus gene sequences into the VR-1012 expression vector produced by Vical, Inc. (San Diego, CA) (13). The VR-1012 expression vector is very similar to the vector backbone used in a plasmid DNA-based malaria vaccine (29) and a multiclade human immunodeficiency virus (HIV) DNA vaccine that has been tested in humans (11). To generate the vaccine (EBODNA012-00-VP) tested in this clinical trial, Ebola virus GP gene sequences were subcloned into a slightly modified VR-1012 plasmid backbone containing the human T-cell leukemia virus 1 R region translational enhancer for improved expression (2). The CMV/R expression vector has been tested in a clinical trial of a multiclade HIV DNA vaccine (11) and in other candidate vaccines currently undergoing evaluation in clinical studies by the Vaccine Research Center and the Division of AIDS, NIAID, National Institutes of Health.

The DNA plasmids were produced in bacterial cell cultures containing a kanamycin selection medium. The process involved *Escherichia coli* fermentation, purification, and formulation as a sterile liquid injectable dosage form for intramuscular injection. Following growth of bacterial cells harboring the plasmid, the plasmid DNA was purified from cellular components.

The vaccine was produced by Vical, Inc. (San Diego, CA), under current Good Manufacturing Practices conditions and met lot release specifications prior to administration. This naked DNA product involves no lipid, viral, or cellular vector components. A phosphate-buffered saline placebo control, pH 7.2, was

TABLE 1. Demographic characteristics at enrollment

Characteristic and subcategory	No. (%) of subjects with characteristic in dose group				
	2 mg (n = 5)	4 mg (n = 8)	8 mg (n = 8)	Placebo (n = 6)	Overall (n = 27)
Gender					
Male	5 (100.0)	5 (62.5)	5 (62.5)	3 (50.0)	18 (66.7)
Female	0 (0.0)	3 (37.5)	3 (37.5)	3 (50.0)	9 (33.3)
Age[a] (yrs)					
18–20	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	1 (3.7)
21–30	1 (20.0)	3 (37.5)	3 (37.5)	0 (0.0)	7 (25.9)
31–44	4 (80.0)	5 (62.5)	4 (50.0)	6 (100.0)	19 (70.4)
Mean (SD)	34.0 (6.4)	36.4 (8.8)	28.3 (8.4)	34.7 (1.9)	33.1 (7.6)
Range	24–41	24–44	18–43	32–37	18–44
Race					
White	5 (100.0)	8 (100.0)	7 (87.5)	6 (100.0)	26 (96.3)
Black or African American	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)	1 (3.7)
Asian	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
American Indian/Alaskan Native	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Native Hawaiian or other Pacific Islander	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Multiracial	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Other/unknown	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ethnicity					
Non-Hispanic/Latino	5 (100.0)	8 (100.0)	8 (100.0)	5 (83.3)	26 (96.3)
Hispanic/Latino	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (3.7)
BMI[b]					
Under 18.5	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
18.5–24.9	4 (80.0)	6 (75.0)	3 (37.5)	3 (50.0)	16 (59.3)
25.0–29.9	0 (0.0)	1 (12.5)	3 (37.5)	2 (33.3)	6 (22.2)
30.0 or over	1 (20.0)	1 (12.5)	2 (25.0)	1 (16.7)	5 (18.5)
Mean (SD)	23.0 (4.0)	25.8 (7.1)	27.0 (7.9)	25.5 (5.3)	25.6 (6.4)
Range	20.1–30.1	21.0–42.2	19.5–39.2	20.6–34.7	19.5–42.2
Education					
Less than high school graduate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
High school graduate/GED	1 (20.0)	2 (25.0)	2 (25.0)	0 (0.0)	5 (18.5)
College/university	3 (60.0)	3 (37.5)	5 (62.5)	2 (33.3)	13 (48.1)
Advanced degree	1 (20.0)	3 (37.5)	1 (12.5)	4 (66.7)	9 (33.3)

[a] Age at enrollment day.
[b] Height and weight (used for BMI) were from the screening evaluation.

produced under current Good Manufacturing Practices conditions by Bell-More Labs, Inc. (Hampstead, MD).

Measurement of antibody responses: enzyme-linked immunosorbent assay (ELISA). Endpoint titers of antibodies directed against Ebola virus antigens NP (Z), GP (S/G), and GP (Z) were determined using 96-well Immulon 2 plates (Dynex Technologies) coated with a preparation of purified recombinant proteins according to methods adapted from those described previously (11). Biotin-labeled anti-human immunoglobulin G (IgG), IgA, or IgM and streptavidin conjugated with horseradish peroxidase and 3,5′,5,5′-tetramethylbenzidine substrate was used to develop the reaction, which was detected on a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The endpoint titer was calculated as the most dilute serum concentration that gave an optical density reading of >0.2 above background.

Measurement of antibody responses by immunoprecipitation and Western blot analysis. Antibody responses were measured in a semiquantitative assay combining immunoprecipitation (IP) of crude cell-free supernatants containing GP (Z) or GP (S/G) or cell lysates containing NP protein with volunteer sera, followed by Western blotting for GP or NP as previously described (7). Briefly, sera (10 μl) from immunized individuals were used to immunoprecipitate Ebola virus proteins either from 100 μl of cell-free supernatant or from cell lysates of 293 cells (100 μl of cell lysate is equivalent to 300 to 400 μg of total protein) transfected with vectors encoding transmembrane-deleted Ebola virus GP (Z) or transmembrane-deleted Ebola virus GP (S/G) or Ebola virus NP (Z). Immune complexes were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using the following Ebola virus protein-specific antibodies: mouse monoclonal 12B5 (generous gift from Mary Kate Hart, USAMRIID) against GP (Z), rabbit polyclonal B83 (generous gift from Barton Haynes, Duke University) against GP (S/G), or mouse monoclonal 1C9 (generous gift from Barton Haynes, Duke University) against NP. Preimmune sera (10 μl) from those individuals were used as controls. The gels were scanned, and the intensity of each band was quantified by densitometry using the program ImageQuant; results are presented graphically to facilitate comparisons among groups.

Measurement of T-cell responses and cell preparation. Peripheral blood mononuclear cells (PBMC) were prepared by standard Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). PBMC were frozen in heat-inactivated fetal calf serum containing 10% dimethyl sulfoxide in a Forma CryoMed cell freezer (Marietta, OH). Cells were stored at −140°C. All immunogenicity assays were performed on thawed specimens; average viability was >95%.

Antibodies. Unconjugated mouse anti-human CD28, unconjugated mouse anti-human CD49d, allophycocyanin-conjugated mouse anti-human CD3, fluorescein isothiocyanate-conjugated mouse anti-human CD8, peridinin chlorophyll protein-conjugated mouse anti-human CD4, and a mixture of phycoerythrin-conjugated mouse anti-human gamma interferon (IFN-γ) and interleukin 2 (IL-2) monoclonal antibodies were obtained from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA). All reagents were independently titrated to determine the optimum concentrations for staining.

Peptides and cell stimulation. Peptides 15 amino acids in length, overlapping by 11, and corresponding to the vaccine inserts were synthesized at >85% purity as confirmed by high-performance liquid chromatography (24). Peptides were pooled for each protein, NP (Z), GP (S/G), and GP (Z), and used at a final

concentration of 500 ng per stimulation. Cell stimulation was performed as described previously (11). Briefly, one million PBMC in 200 μl R-10 medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 1.7 mM sodium glutamate) were incubated with 1 μg/ml each of costimulatory anti-CD28 and -CD49d monoclonal antibodies and 2.5 μg/ml of each peptide in wells of 96-well V-bottom plates. Cells incubated with only costimulatory antibodies were included in every experiment to control for spontaneous production of cytokine and activation of cells prior to addition of peptides. Staphylococcal enterotoxin B (10 μg/ml; Sigma-Aldrich) was used as a positive control for lymphocyte activation. Cultures were incubated at 37°C in a 5% CO_2 incubator for 6 h in the presence of brefeldin A (10 μg/ml; Sigma, St. Louis, MO).

Intracellular cytokine and immunofluorescence staining. Cells were permeabilized for 7 min in 200 μl of a solution containing 67 μl Tween 20 (Sigma), 106 μl deionized water, and 27 μl of 10× FACS-Lyse solution (BDIS) at room temperature, washed twice in cold Dulbecco's phosphate-buffered saline containing 1% fetal bovine serum and 0.02% sodium azide (FACS [fluorescence-activated cell sorting] buffer), and stained directly with conjugated anti-human CD3, anti-human CD4, anti-human CD8, and anti-human IFN-γ and IL-2 antibodies for 15 min on ice. Stained cells were then immediately washed twice with cold FACS buffer. The cells were resuspended in Dulbecco's phosphate-buffered saline containing 1% paraformaldehyde (Electron Microscopy Systems, Fort Washington, PA) and stored at 4°C until analysis. Four-parameter flow cytometric analysis was performed on a FACSCalibur flow cytometer (BDIS). Following intracellular cytokine staining (ICS), between 50,000 and 250,000 events were acquired, gated on small lymphocytes, and assessed for CD3, CD8, CD4, and IFN-γ/IL-2 expression. Results were analyzed using FlowJo software (Tree Star Software, Ashland, OR). The same cytokine, CD4, and CD8 gates were used for the entire trial.

ELISPOT. Vaccine-induced T-cell responses were also detected by enzyme-linked immunospot assays (ELISPOT) according to a modification of previously published methods (11) using a commercially available ELISPOT kit (BD Biosciences). PBMC were stimulated overnight at 37°C in triplicate wells at a density of 2×10^5 cells/well for all stimulations other than staphylococcal enterotoxin B, which was conducted at 5×10^4 cells/well. Following incubation, cells were lysed, and the wells were washed and incubated for 2 h at room temperature in the presence of biotinylated IFN-γ detection antibodies. Subsequently, the wells were incubated with an avidin-horseradish peroxidase solution for 1 h at room temperature, followed by a 20-min incubation with the AEC substrate solution. The plate was air dried for a minimum of 2 hours prior to spot quantitation on a CTL ELISPOT image analyzer (Cellular Technology Ltd., Cleveland, OH). Results were expressed as mean spot-forming cells per million PBMC.

Statistical methods. Positive response rates to any antigen (GP [S/G], GP [Z], or NP [Z]) and to each individual antigen were used to summarize the T-cell response data; exact two-sided 95% confidence intervals (29) are reported. The positivity criteria for the ICS data consisted of a statistical hypothesis test for a difference in the stimulated and unstimulated wells followed by the requirement of a minimal level of response. For an individual's response to be categorized as positive, it had to be statistically significant and had to exceed the threshold for positivity. Positivity thresholds were based on an ICS validation study of HIV peptides completed at the VRC. The thresholds were selected to give a 1% false-positive rate across PBMC from 34 HIV type 1-seronegative individuals stimulated with eight HIV peptide pools in the validation data set. Only 2 of the 272 samples (0.007) had responses exceeding the thresholds. The validation study results using HIV peptides are expected to be relevant for the Ebola virus peptides; hence, in addition to the statistical hypothesis test for positivity, the same thresholds were used. For the ICS responses, Fisher's exact test was applied to each antigen-specific response versus the negative control response, with a Holm adjustment for the multiple comparisons. The nominal significance level was $\alpha = 0.01$, and the minimum threshold for background-corrected percent positive response was 0.0241 for $CD4^+$ and 0.0445 for $CD8^+$. To determine positivity of the ELISPOT responses, a permutation test was applied to each antigen-specific response versus negative control responses using the Westfall-Young approach to adjust for the multiple comparisons. The nominal significance level was $\alpha = 0.05$. In addition, for the sample to be categorized as positive, the result had to achieve a statistically significant difference and be above a predetermined cutoff set at a false-positive rate of <1% (i.e., the mean difference in the antigen-stimulated wells and the negative control wells had to be greater than or equal to 10 spot-forming cells per 2×10^5 PBMC). A variance filter for the antigen-specific responses was also used: samples with a ratio of antigen-well variance (median, +1) greater than or equal to 100 were discarded from the analysis; no such samples were found in the data set. SAS (version 9.1; SAS Institute) and Splus (version 6.0; Insightful) were used for all analyses.

TABLE 2. Local and systemic reactogenicity[a]

Symptom and intensity	No. (%) of patients with reaction in dose group			
	2 mg ($n = 5$)	4 mg ($n = 8$)	8 mg ($n = 8$)	Placebo ($n = 6$)
Local symptoms				
Pain or tenderness				
None	0 (0.0)	1 (12.5)	2 (25.0)	3 (50.0)
Mild	5 (100.0)	5 (62.5)	3 (37.5)	3 (50.0)
Moderate	0 (0.0)	2 (25.0)	3 (37.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Induration				
None	3 (60.0)	4 (50.0)	2 (25.0)	6 (100.0)
Mild	2 (40.0)	4 (50.0)	6 (75.0)	0 (0.0)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Skin discoloration				
None	2 (40.0)	1 (12.5)	2 (25.0)	5 (83.3)
Mild	3 (60.0)	7 (87.5)	6 (75.0)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Any local symptom				
None	0 (0.0)	1 (12.5)	1 (12.5)	3 (50.0)
Mild	5 (100.0)	5 (62.5)	4 (50.0)	3 (50.0)
Moderate	0 (0.0)	2 (25.0)	3 (37.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Systemic symptoms				
Malaise				
None	4 (80.0)	6 (75.0)	3 (37.5)	4 (66.7)
Mild	1 (20.0)	0 (0.0)	4 (50.0)	0 (0.0)
Moderate	0 (0.0)	2 (25.0)	0 (0.0)	2 (33.3)
Severe[b]	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Myalgia				
None	5 (100.0)	7 (87.5)	5 (62.5)	6 (100.0)
Mild	0 (0.0)	1 (12.5)	3 (37.5)	0 (0.0)
Moderate	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Headache				
None	4 (80.0)	5 (62.5)	6 (75.0)	5 (83.3)
Mild	1 (20.0)	3 (37.5)	1 (12.5)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Nausea				
None	4 (80.0)	6 (75.0)	3 (37.5)	5 (83.3)
Mild	1 (20.0)	2 (25.0)	4 (50.0)	1 (16.7)
Moderate	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Fever				
None	5 (100.0)	7 (87.5)	8 (100.0)	6 (100.0)
Mild	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Moderate	0 (0.0)	1 (12.5)	0 (0.0)	0 (0.0)
Severe	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Any systemic symptom				
None	4 (80.0)	5 (62.5)	3 (37.5)	3 (50.0)
Mild	1 (20.0)	1 (12.5)	3 (37.5)	1 (16.7)
Moderate	0 (0.0)	2 (25.0)	1 (12.5)	2 (33.3)
Severe[b]	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)

[a] The local injection site reactions were recorded by clinicians at 30 to 45 min postinjection and were then recorded as self-assessments at home by subjects on a 7-day diary card. Systemic reactions were recorded as self-assessments at home by subjects on a 7-day diary card following each injection.

[b] A single severe systemic symptom (malaise) was related to a foot fracture which occurred 6 days following vaccination.

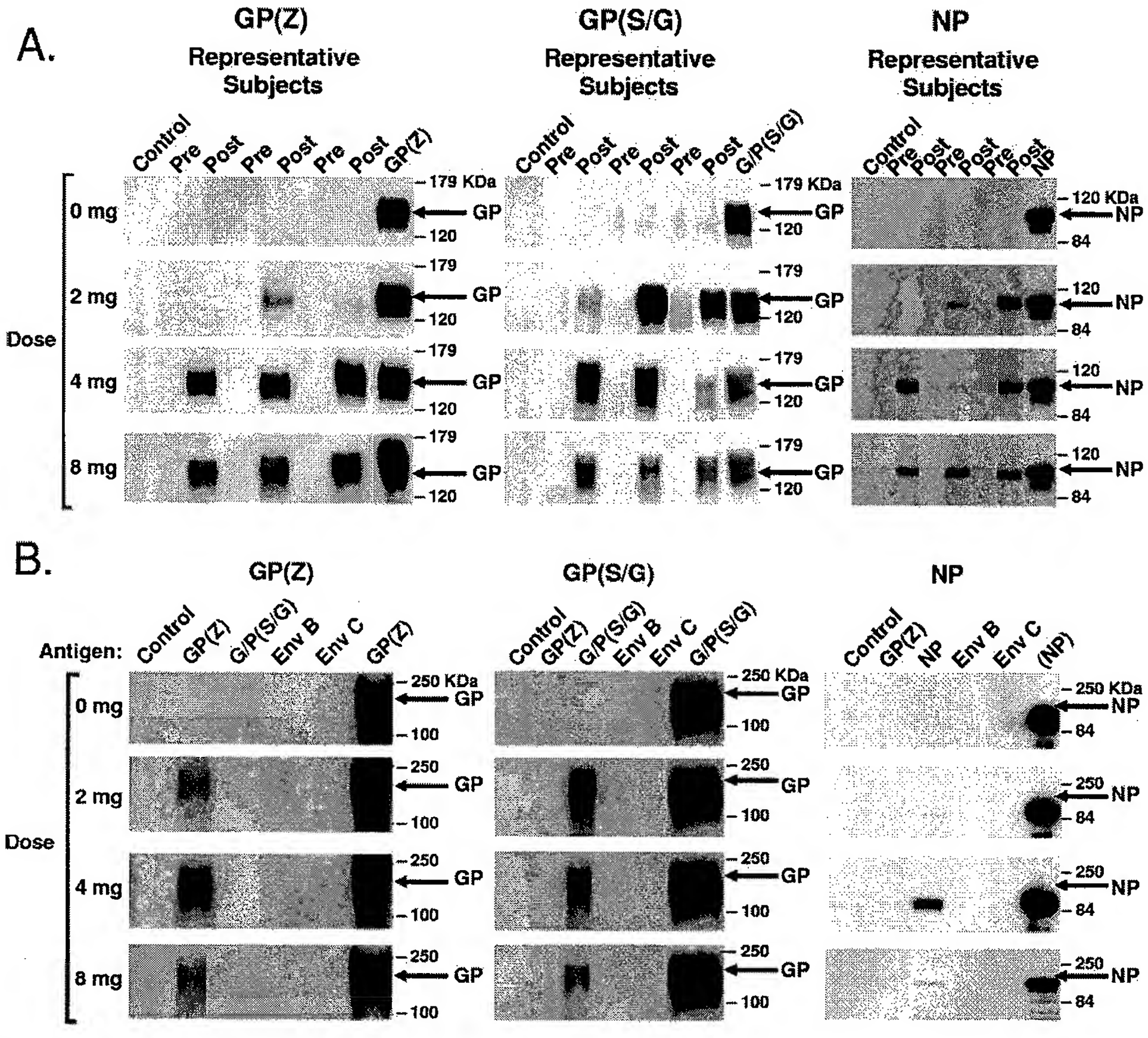

FIG. 1. Specific antibody responses to all vaccine components by IP-Western blot analysis. Sera from three representative subjects from each vaccine dose group are shown for each antigen (A). Sera were drawn at week 12, 4 weeks following the third vaccination. Antibody responses were specific and not cross-reactive to other vaccine antigens based on immunoblotting with monoclonal antibodies (B).

RESULTS

Study population demographics. A total of 27 healthy adult volunteers were enrolled, with 5 in the 2-mg dose group, 8 each in the 4-mg and 8-mg dose groups, and 6 in the placebo group. Table 1 includes demographic data regarding subject gender, age, race/ethnicity, body mass index (BMI), and educational level at the time of enrollment. The subject population was 66.7% male and 33.3% female with a mean age of 33.1 years (range of 18 to 44 years). Subjects were predominantly white (96.3%) and non-Hispanic/Latino (96.3%). The mean BMI was 25.6 (range, 19.5 to 42.2). All subjects had an educational level of high school or higher, with 48.1% having college level degrees and 33.3% holding advanced degrees.

Vaccine safety. Due to a theoretical concern over GP-mediated cytopathicity (22), coagulation parameters of study subjects were closely monitored. At enrollment and throughout the study, D-dimer, prothrombin time, partial thromboplastin time, fibrinogen, complete blood count, and red blood cell smears were evaluated. There were no reportable coagulation laboratory abnormalities.

Two subjects were withdrawn from the vaccination schedule due to serious adverse events that were assessed as "possibly" related to vaccination: a grade 4 creatine phosphokinase elevation 2 weeks after first vaccination and a grade 2 herpes zoster thoracic dermatome eruption 3 weeks after the second vaccination, both in 8-mg recipients. Of note, the grade 4 creatine phosphokinase elevation was associated with vigorous exercise. These events resolved without sequelae, and these subjects continued to participate in the study and attended all study visits. Although only six of eight subjects in the 8-mg dose group received all three injections, the immunogenicity and safety laboratory values for all subjects are included in the

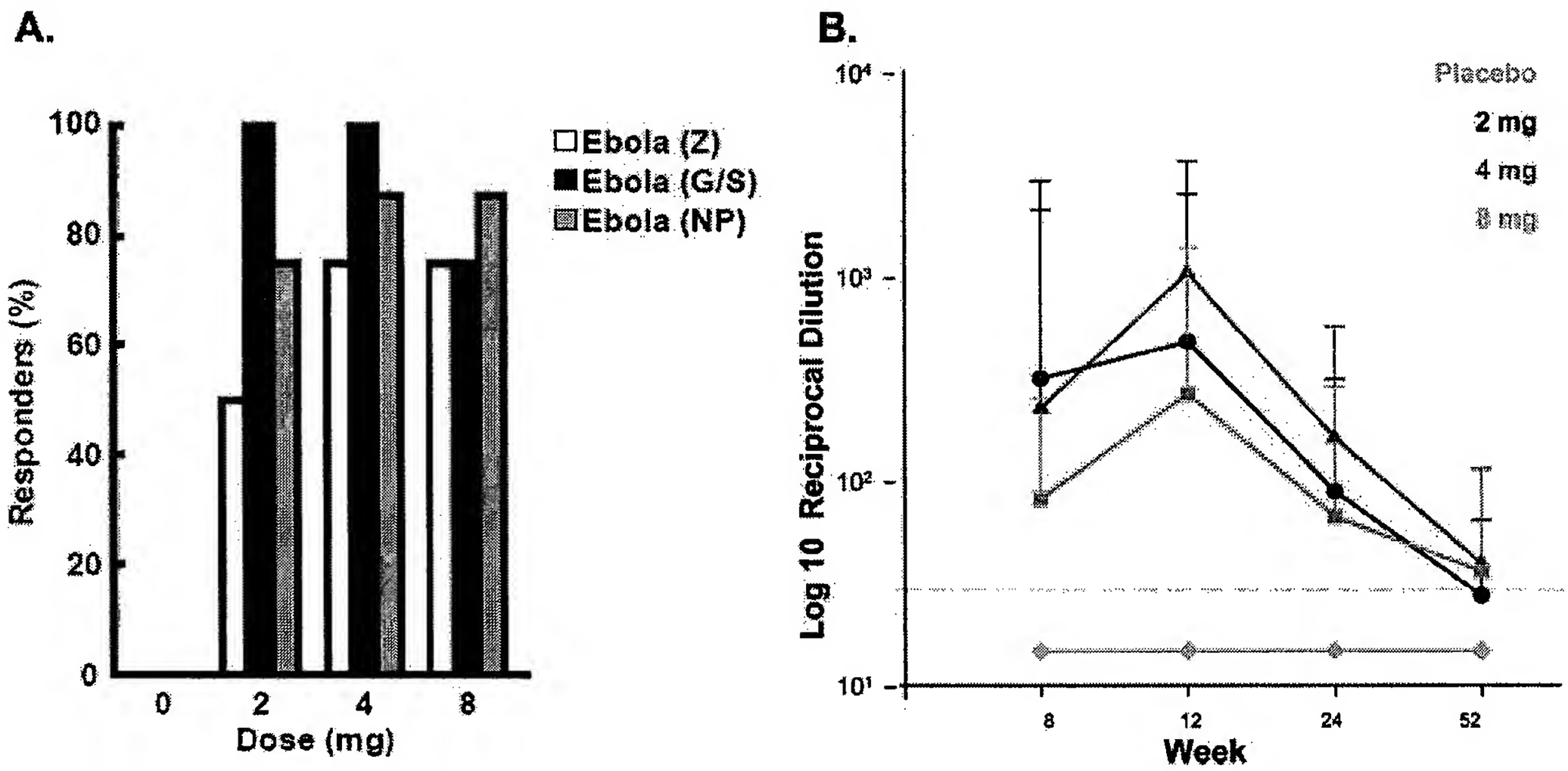

FIG. 2. Kinetics and frequency of antibody responses. (A) Percentages of responders following the third vaccination by the IP-Western assay for all subjects are shown. The *y* axis represents the percentage of responders with a positive assay, and the *x* axis represents the vaccine dose group. White bars, GP (Z); black bars, GP (S/G); gray bars, NP (Z). (B) Kinetics of the antibody response for all subjects is shown over the 52 weeks of the study. The geometric mean titer of the $\log_{10}$ reciprocal dilution and standard deviation of the antibody response to GP (S/G) are plotted against the number of weeks after initial vaccination for each of the three dose levels. Vaccinations were given at 0, 4, and 8 weeks. The threshold for positivity in this assay was a reciprocal dilution of 30 and is shown as a dashed line. Of note, only six of eight subjects in the 8-mg dose group received all three vaccinations in the series, yet all vaccinees are included in the immunogenicity analysis.

analyses. One subject in the 2-mg dose group chose to withdraw after the second vaccination; another subject (in the placebo group) withdrew after the third injection. Neither of these subjects returned for further visits and, therefore, they were not included in the immunogenicity analysis due to a lack of samples at time points following their withdrawal. As a result, 20 of 21 vaccinees had immune responses assessed. All subjects are represented in the safety data through the time points available.

The diary cards showed that 90.5% (19/21) of subjects who received vaccine (at any dose level) experienced at least one local injection site symptom (mild to moderate pain/tenderness, mild induration, or mild skin discoloration) following a vaccination. The systemic symptoms recorded on diary cards included malaise, myalgia, headache, nausea, and fever, as well as local injection site symptoms (Table 2). The study vaccinations were well-tolerated and safe in healthy subjects, ages 18 to 44 years.

Antibody responses. Ebola virus-specific humoral responses were detected in all vaccinees. GP- and NP-specific antibody responses were detected by IP-Western blot analysis (Fig. 1). Initial analysis of three representative subjects at different vaccine doses, 4 weeks following the third dose of vaccine (week 12), revealed antibodies specific for GP (Zaire) or GP (Sudan/Gulu) (Fig. 1A, left and middle panels) and to NP (Fig. 1A, right panel). These data demonstrate that specific antibodies to each antigen can be induced by the vaccine independently and are not cross-reactive (Fig. 1B). All (100%) of the 2-mg and 4-mg recipients and 75% (6/8) of the 8-mg recipients made GP antibodies. Three-fourths (75%) of the 2-mg and 87.5% of the 4-mg and 8-mg recipients produced an NP-specific antibody response (Fig. 2A). All (100%) vaccinees made a specific antibody response detected by ELISA to at least one of the three antigens encoded by the vaccine, with 19 of 20 vaccinees producing a GP (Z)- and GP (S/G)-specific antibody response at one or more time points (data not shown). This antibody response was detected after the second dose of vaccine in some subjects, peaked after the third dose (week 12), and waned over the course of 1 year (Fig. 2B). Antibody titers (reciprocal dilution) at week 12 ranged from undetectable to 4,000 for either GP antigen (see Tables S1 and S2 in the supplemental material). Ebola virus-specific neutralizing antibody, measured by a pseudotyped virus neutralization assay (23), was not detected in any study subject (data not shown), as might be expected with DNA vaccination in the absence of rAd boosting.

T-cell responses. $CD4^+$ and $CD8^+$ T-cell responses were assessed by ICS for all three antigens encoded by the vaccine for all study subjects. GP (S/G) was the stronger T-cell immunogen of the two GP antigens encoded by the vaccine, and NP induced the weakest response of the three antigens but was still measurable in the majority of vaccinees. An Ebola virus-specific $CD4^+$ T-cell response was demonstrated in all vaccinees by ICS, and many of these responses occurred by week 4, following just one dose of vaccine. $CD4^+$ T-cell responses for GP (S/G) were detected in 100% of vaccinees by week 10. By week 12, 100% of 2-mg (4/4) and 88% of 4-mg (7/8) and 8-mg (7/8) recipients produced a $CD4^+$ T-cell GP (Z)-specific response; by week 52, 100% of 2-mg (4/4) and 4-mg (8/8) recip-

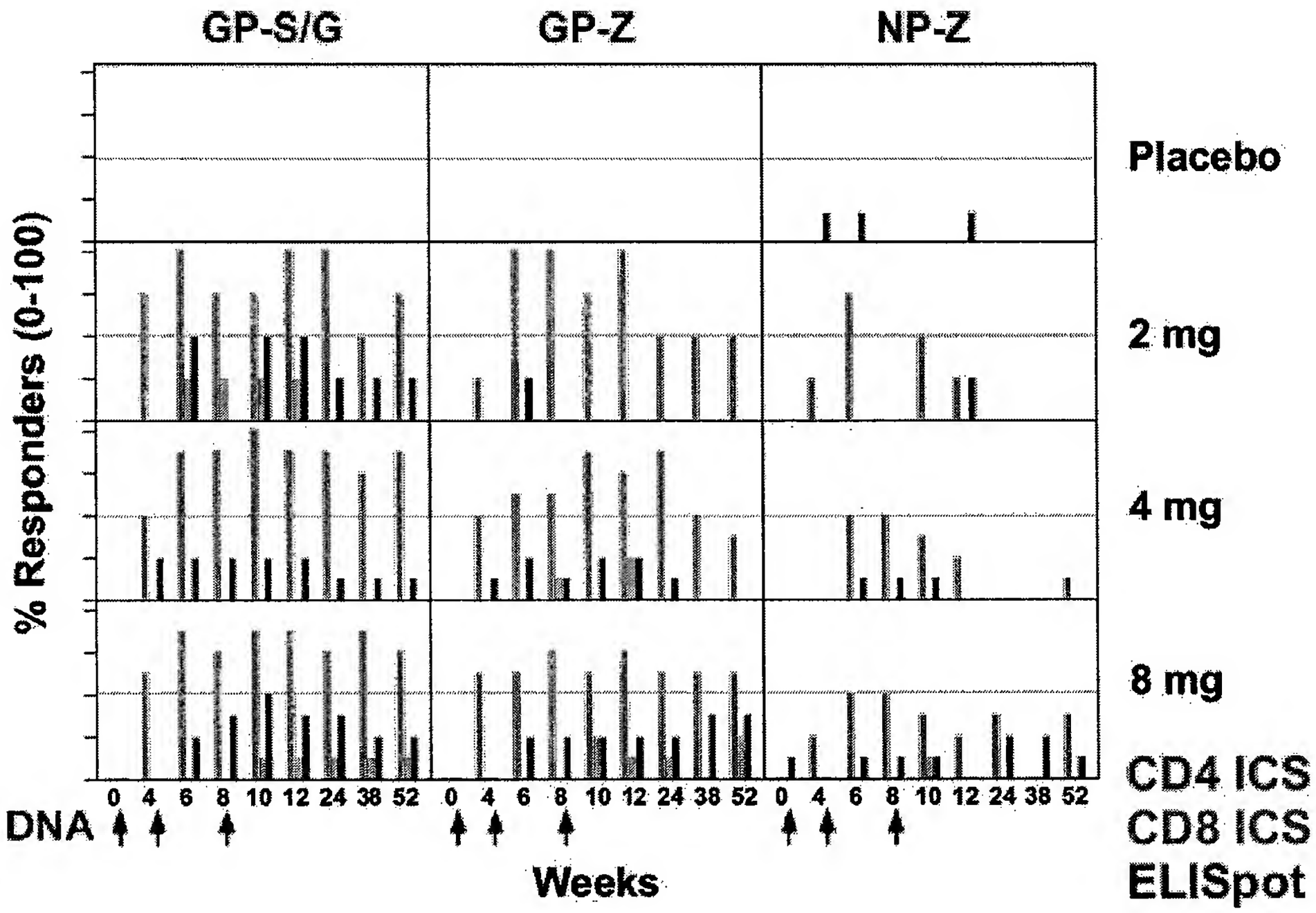

FIG. 3. Frequency of $CD4^+$ and $CD8^+$ T-cell responses by ICS and ELISPOT analysis. Frequency (percent responders) is represented on the left *y* axis. The week of analysis is shown on the lower *x* axis, the antigen assessed is shown on the upper *x* axis, and vaccine dose group is shown on the right *y* axis. The frequency of $CD4^+$ ICS responses is shown by red bars, the frequency of $CD8^+$ ICS responses is shown by green bars, and the frequency of positive ELISPOT responses is shown with blue bars. The schedule of the three DNA vaccinations is represented by arrows along the lower *x* axis.

ients and 88% (7/8) of 8-mg recipients produced a $CD4^+$ T-cell response to the GP (Z) antigen. By week 10, 75% (3/4) of 2-mg and 4-mg recipients (6/8) and 100% of 8-mg (8/8) recipients developed a $CD4^+$ T-cell response to NP (Fig. 3).

$CD8^+$ T-cell Ebola-specific responses were detected less frequently than $CD4^+$ T-cell responses but were present in 30% (6/20) of all vaccinees by ICS. By week 10, 25% (1/4) of 2-mg, none of the 4-mg, and 13% (1/8) of 8-mg recipients produced a $CD8^+$ T-cell response to GP (S/G). By week 12, none of the 2-mg and 25% (4/16) of the 4-mg and 8-mg recipients generated a $CD8^+$ T-cell response to GP (Z). None of the 2-mg or 4-mg vaccinees and only one of the 8-mg vaccinees produced a measurable $CD8^+$ T-cell response to the NP antigen by week 10 as assessed by ICS (Fig. 3).

Analyses were also performed on all study subjects for all three antigens by ELISPOT. Consistent with the ICS results, the dominant antigen was GP (S/G). By week 12, 50% (2/4) of 2-mg, 63% (5/8) of 4-mg, and 63% (5/8) of 8-mg recipients developed a positive ELISPOT response to GP (S/G). By week 12, 50% of 2-mg (2/4), 50% of 4-mg (4/8), and 38% of 8-mg (3/8) recipients had a positive ELISPOT response to GP (Z) as well. By week 24, 25% (1/4) of 2-mg, 13% (1/8) of 4-mg, and 63% (5/8) of 8-mg recipients displayed positive ELISPOT responses to the NP antigen (Fig. 3).

The magnitude of the $CD8^+$ T-cell response was slightly less than that seen in the $CD4^+$ T-cell analysis as assessed by ICS. The GP (S/G) immunogen induced slightly higher-magnitude $CD4^+$ T-cell responses compared to the other immunogens in the vaccine. The magnitude of the GP (Z)-specific $CD4^+$ response was 70% of the GP (S/G) response, while the magnitude of the NP-specific $CD4^+$ response was 16% of the GP (S/G) response. A correlation was not seen in the low number of positive responses as assessed by ICS for $CD8^+$ T cells (Fig. 4). Analysis of the kinetics of the T-cell responses revealed that the responses peaked between weeks 10 and 12 and, in general, detectable responses were not sustained, although there was a trend in the higher dose group toward a slightly greater duration of detectable responses (Fig. 5). Consistent with the ICS and antibody responses, the ELISPOT response was of greatest magnitude for the GP (S/G) antigen (see Table S3 in the supplemental material).

DISCUSSION

The rapid progression of severe disease after Ebola virus infection allows little opportunity to develop protective immunity, and there is currently no effective antiviral therapy. Therefore, vaccination offers a promising intervention to pre-

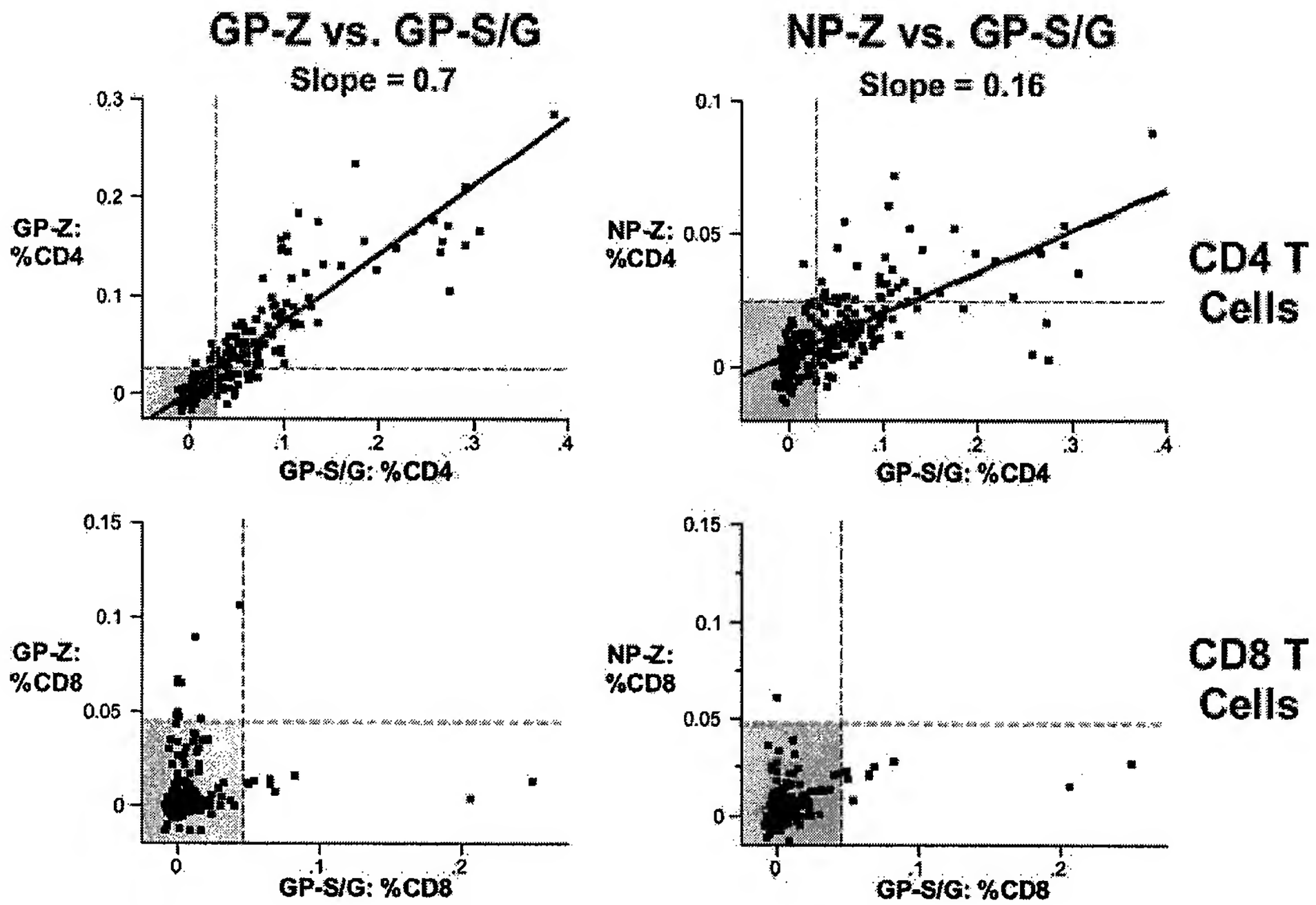

FIG. 4. Magnitude of antigen-specific T-cell responses to vaccine components. GP (S/G) (*x* axes) are shown in relation to each of the other two antigens, GP (Z) and NP (Z) (*y* axes). All antigen-specific $CD4^+$ and $CD8^+$ T-cell responses for all subjects (vaccine and placebo recipients) as assessed by ICS are shown. $CD4^+$ and $CD8^+$ T-cell responses are shown as a percentage of total $CD4^+$ or $CD8^+$ T cells on the *x* and *y* axes. CD4 responses are shown in the upper graphs, and $CD8^+$ responses are shown in the lower graphs. The red dashed line represents the threshold of positivity (0.0445% for $CD8^+$ T cells and 0.0241% for $CD4^+$ T cells).

vent infection or severe disease and limit spread to contacts and would be an important public health benefit for health care workers involved in the care of patients and containment of outbreaks. Another compelling reason for accelerated development of an Ebola virus vaccine relates to its contribution to biodefense (17). The Centers for Disease Control and Prevention (6) Category A agents are highly contagious and largely lack effective vaccines or treatments (18) and include the filoviruses (Ebola and Marburg viruses).

Gene-based vaccine technology provides a safe avenue for producing candidate vaccines for select agents without the need for extreme biocontainment. Gene-based vectors for filoviruses are particularly attractive vaccine approaches because of their capacity to induce both humoral and cell-mediated immune responses, both of which may be important for protection. The concept of using bacterium-derived plasmid DNA to deliver vaccine antigens has many attractive features, including (i) ease and flexibility of construction, (ii) scalable manufacturing capacity, (iii) stability, (iv) intracellular production of vaccine antigen, (v) transient expression, (vi) no induction of antivector immunity, (vii) induction of both $CD4^+$ and $CD8^+$ T-cell responses as well as antibody, and (viii) lack of local or systemic reactogenicity. However, DNA vaccines have not performed well enough to be considered as a vaccine platform in humans until recently. A hepatitis B virus DNA vaccine administered by a needle-free particle-mediated delivery was shown to be safe and immunogenic in a phase I clinical trial (20). Additionally, DNA vaccination against malaria was shown to be safe and immunogenic, especially as a priming vaccination in a prime-boost regimen (16, 30). Recently, a multiclade HIV DNA vaccine based on a similar design to the Ebola virus DNA vaccine described here was shown to be safe and immunogenic in healthy adults (11).

The broad immunogenicity of this Ebola virus DNA vaccine suggests that immunization by plasmid DNA delivery is a viable platform and merits further development. The consistent immunogenicity of the Ebola virus DNA vaccine described here likely reflects a combination of factors, including optimization of vector design, manufacturing methods, delivery, sample processing, and immunological assays. Additional work is needed to further improve the efficiency and consistency of DNA vaccination.

Nonhuman primate studies have shown that an rAd5 vaccine effectively prevents disease, and DNA vaccination prior to boosting with rAd5 also confers protection and markedly increases the magnitude of the immune response (22, 24, 25). Further vector and construct optimization may further increase protective immunity of this DNA vaccine. Recently, the importance of the GP transmembrane region in the design of the immunogen has been described. Reduced protection with

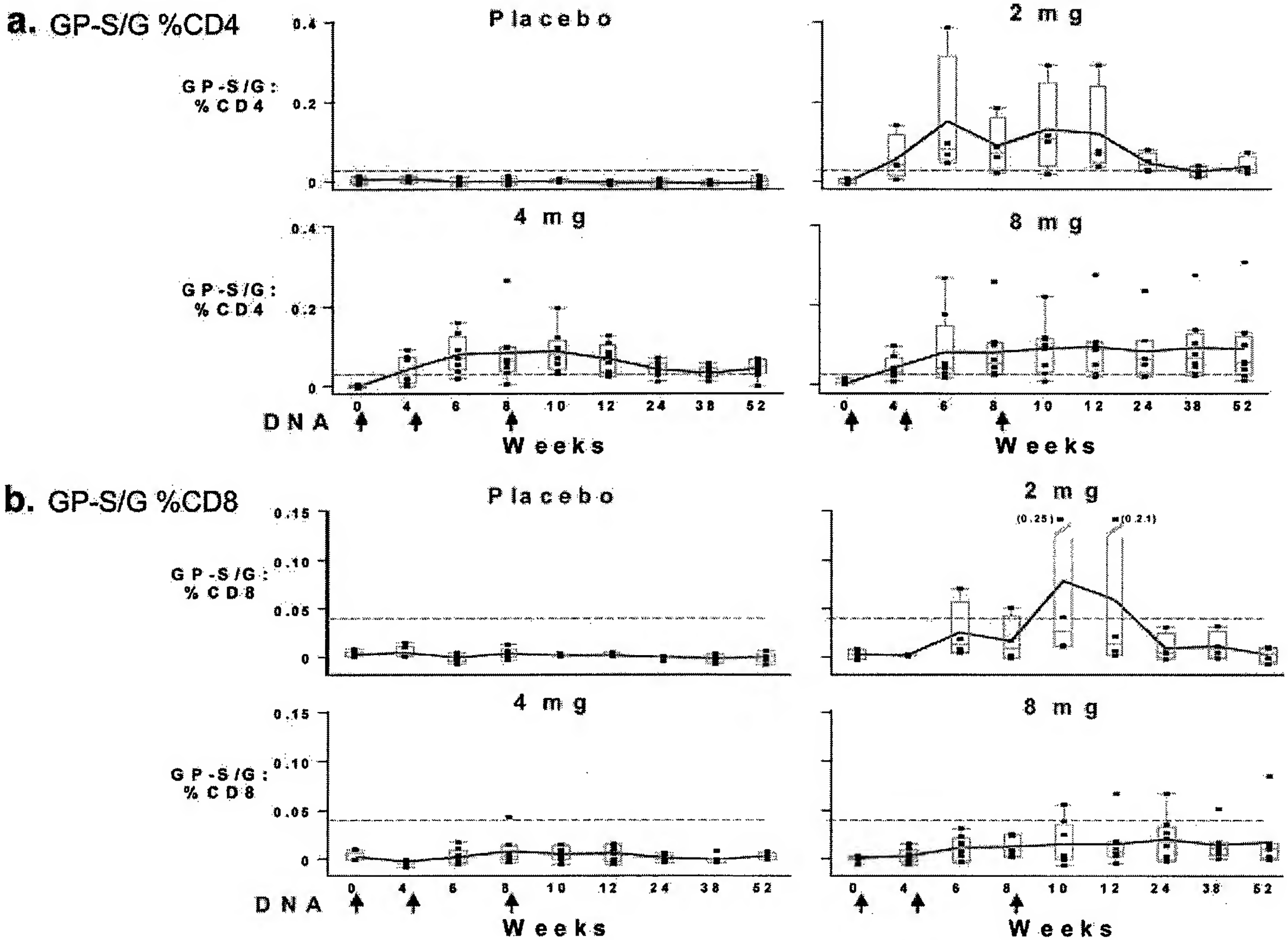

FIG. 5. Kinetics of $CD4^+$ and $CD8^+$ T-cell responses to GP (S/G). Responses were assessed by ICS and are shown over the course of the study. Results are presented by dose group, with a blue line indicating the mean response over time for all subjects in a given group. The median of the response is represented in the box and whisker plots at each time point. The red dashed lines represent the positivity threshold for the ICS assay.

an Ebola virus rAd immunogen containing a GP transmembrane region deletion compared to a point mutation in this region or wild-type GP constructs has been found (22). Additionally, it was found that the NP gene is dispensable for immune protection, and the addition of NP in a candidate vaccine may diminish the immune response to Ebola virus GP (23). Therefore, future formulations of this DNA product will include multiple GP constructs encoding GP in either its wild-type form or a modified form to optimize vaccine potency. Because Ebola virus from Ivory Coast has been observed in only one limited outbreak and is closely related to Ebola virus Zaire, it is not included in vaccine formulations.

This is the first report of an evaluation of a candidate Ebola virus vaccine in humans. This three-plasmid DNA candidate Ebola virus vaccine was safe and well-tolerated in 21 healthy adults. Importantly, DNA immunization induced both Ebola virus-specific antibody and T-cell responses to the GP and NP antigens. While Ebola virus-specific neutralizing antibody could not be detected in vaccinees, the range of antibody titers measured by ELISA was similar to those seen in nonhuman primates following vaccination with similar vaccine constructs (24). Recently, in a series of nonhuman primate studies demonstrating protection from Ebola virus with vaccine constructs expressing similar antigens as used in this clinic trial, IgG as measured by ELISA correlated with survival. In functional assays, serum antibodies were neither neutralizing nor enhancing, suggesting that IgG levels may reflect the overall level of immune stimulation. Although antibody-dependent enhancement of Ebola virus replication has been observed in tissue culture, there is no evidence of antibody-dependent enhancement in humans or in animal studies, and only protection, rather than enhancement, has been observed in animal studies evaluating DNA or rAd-based vaccine strategies (23, 27). In the clinical trial described here, the vaccine-induced antibody and T-cell-mediated immune responses were greatest to the GP immunogens, with a less frequent response to the NP immunogen, and Ebola virus-specific $CD4^+$ T-cell responses were more frequent than $CD8^+$ T-cell responses. While the presence of Ebola virus GP-specific IgG seems to predict survival in nonhuman primates, the definite correlate(s) of protection from Ebola virus infection is not known, and it is possible that T-cell responses also contribute to protection. Therefore, we believe it is important that a candidate Ebola virus vaccine be capable of eliciting both Ebola virus-specific antibody and T-cell responses.

Further studies are needed to determine the optimal preventive gene-based Ebola virus vaccine strategy. Our development plan includes evaluation of DNA vaccination alone, rAd5 vaccination alone, and a heterologous prime-boost strategy of DNA priming followed by rAd boosting. Even if the optimal strategy were determined to be heterologous prime-boost, the potential vaccines would need to be independently demonstrated as safe and immunogenic. Since the prophylactic efficacy of an Ebola virus vaccine cannot feasibly or ethically be demonstrated in a human trial, the combination of safety and immunogenicity data from phase I, II, and III human trials and efficacy data from nonhuman primate studies will ultimately need to be utilized to obtain licensure of an Ebola virus vaccine under the Animal Rule.

The successful evaluation of a DNA vaccine to multiple Ebola virus subtypes reported here provides the opportunity for further clinical evaluation of candidate Ebola virus DNA vaccines alone or in combination with Ebola virus rAd vaccines as a heterologous prime-boost strategy. Evaluation of gene-based candidate vaccines in humans will continue in parallel with efforts to define immunological correlates of vaccine-induced protection in nonhuman primate models of Ebola virus infection. Together, these studies will provide the scientific basis for identifying a vaccine strategy for the prevention of Ebola virus and other filovirus infections in humans.

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EXHIBIT

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SYMPOSIUM PAPER

The "A, B and C" of Her-2 DNA vaccine development

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Abstract
Introduction The development of Her-2 DNA vaccine has progressed through three phases that can be categorized as phase "A": the pursuit of Her-2 as a tumor-associated "antigen", phase "B": tilting the "balance" between tumor immunity and autoimmunity and phase "C": the on-going "clinical trials".
Materials and methods In phase "A", a panel of human ErbB-2 or Her-2 plasmids were constructed to encode non-transforming Her-2 derivatives. The immunogenicity and anti-tumor activity of Her-2 DNA vaccines were tested in human Her-2 transgenic mice with or without the depletion of regulatory T cells (Tregs). However, Treg depletion or other immune modulating regimens may increase the risk of autoimmunity. In phase "B", the balance between tumor immunity and autoimmunity was assessed by monitoring the development of experimental autoimmune thyroiditis (EAT). To test the efficacy of Her-2 DNA vaccines in cancer patients, clinical trials have been initiated in phase "C".
Results and conclusions Significant anti-Her-2 and anti-tumor activity was observed when Her-2 transgenic mice were electro-vaccinated after Treg depletion. Susceptibility to EAT was also enhanced by Treg depletion and there was mutual amplification between Her-2 immunity and EAT development. Although Tregs regulate both EAT and Her-2 immunity, their effector mechanisms may differ. It may be possible to amplify tumor immunity with improved strategies that can by-pass undue autoimmunity. Critical information will be revealed in the next decade to expedite the development of cancer vaccines.

Keywords Her-2 · ErbB-2 · DNA vaccine · Cancer vaccine · Autoimmunity

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Introduction

The development of a cancer vaccine generally progresses through three phases that can be categorized as phases "A, B and C". In phase "A", the effort is focused on the "antigen" of interest. Much debate goes into choosing the target molecule, whereas the vaccine formulation may be based on rational design or technical feasibility. An appropriate animal model is a must and determines the validity of the experimental approach. A less considered, but equally important issue is the "balance" between the induction of tumor immunity and autoimmune diseases or phase "B" of the study. Since tolerance to tumor associated antigen (TAA) is profound, strenuous immune modulation is required to achieve meaningful response. A predictable side effect of such immune modulation is inadvertent reactivity to self-antigens. Yet, the balance between tumor immunity and autoimmunity is often considered in a cursory manner in pre-clinical studies and autoimmune side effects are treated symptomatically in patients undergoing immunotherapy. When a "clinical trial" of the candidate vaccine is initiated in phase "C", it can mark the beginning or the end of the effort. In this presentation, the "A, B and C" of Her-2

DNA vaccine development is summarized with our current perspective.

Phase A: Her-2 as the vaccine antigen

In the late 1990s, we chose Her-2 as our vaccine target because of the amplified expression on tumor cells and oncogenic activity [1–5]. This choice is now supported by the efficacy of anti-Her-2 mAb Trastuzumab (Herceptin) in Her-2 positive breast cancer therapy [6]. Naked DNA was selected as the vaccine formulation because of its stable and well-defined nature and because humoral and cellular immune responses to the entire repertoire of antigenic epitopes could be induced. Since it is free of confounding foreign entities, DNA vaccines can be administered repeatedly without losing activity. The feasibility of producing large quantities of pure DNA with simple tools is another major advantage. From intramuscular injection of Her-2 DNA, the vaccine regimen has evolved to include both Her-2 and cytokine DNA administered together by electroporation after regulatory T cell depletion [5, 7–16]. The activity of this improved regimen exceeded our initial expectation and Her-2 DNA vaccine is showing promise as a clinical vaccine candidate.

Her-2 DNA vaccine constructs

A panel of human ErbB-2 (E2) DNA constructs have been generated to encode recombinant Her-2 proteins that are free of tyrosine kinase activity and traffic to specific subcellular compartments [5, 7, 17]. Of the four basic constructs, pE2A encodes the full length Her-2 with a single substitution of aa Lys753 to Ala753 to remove the ATP binding lysine residue. pE2TM encodes the signal peptide, extracellular and transmembrane domains without the intracellular domain (ICD). psecE2 encodes the N-terminus aa 1–505 of the extracellular domain as a secreted protein. These three constructs induced both cellular and humoral immune responses and strong protective immunity in BALB/c and C57BL/6 mice. Another construct pcytE2, with truncated ER signal peptide, directs the synthesis of a full-length, short-lived cytoplasmic protein, which is promptly degraded by the proteasome [7]. Accordingly, immunization with pcytE2 activates CD8 T cells without humoral response. In the early studies, Her-2 DNA alone was administered intramuscularly (i.m.) [5, 7, 17]. Later, pGM-CSF was added to the formulation and electroporation was administered at the vaccination site to enhance gene expression and immune reactivity [15, 16, 18].

Although significant Her-2 immunity with complete tumor rejection was achieved in wild type mice, it was necessary to further evaluate vaccine activity in animals that express human Her-2 as a self-antigen to mimic immune tolerance in humans.

Her-2 transgenic mice

Several transgenic mouse strains that express either wild type or mutant rat neu had been developed [19, 20]. Before human Her-2 transgenic mice were available, rat neu transgenic mice were the next best choice and tumors derived from these mice continue to serve important functions in biological studies. Assessment of reactivity against neu induced tumors in neu transgenic mice induced by heterologous human Her-2 vaccine [16], however, may not mimic the events in humans and the results may even be misleading.

To test Her-2 vaccines in a more relevant system, Her-2 transgenic mice were generated in our lab using whey acid protein (WAP) promoter to drive the wild type human ErbB-2 gene [11]. The mice have been back-crossed into C57BL/6 background and are maintained as herterozygotes because homozygous pups are never born. Spontaneous tumors have not been detected in any organs. C57BL/6 Her-2 transgenic mice produce little to no antibodies (Ab) or T cells when immunized with Her-2 DNA, showing their profound immune tolerance to Her-2 [11]. This poor responsiveness verifies Her-2 as a self-antigen and these mice are invaluable for testing candidate Her-2 vaccines. Because these mice do not develop spontaneous tumors, syngeneic tumors expressing human Her-2 by transfection are tested in Her-2 transgenic mice. Additional backcrosses into other mouse strains are underway and their immune responses to Her-2 DNA vaccine are being investigated.

On the occasions when Her-2/neu induced spontaneous mammary tumors are required, we use tumors from BALB NeuT mice, which express a transforming rat neu and develop spontaneous mammary tumors between 17 and 19 weeks of age. When it is necessary to test DNA vaccines in these mice, rat neu DNA is used. Several mammary tumor lines have been derived from NeuT spontaneous tumors, such as TUBO [20] and Bam1a [21] which grow progressively in NeuT as well as normal BALB/c mice. These cell lines are convenient test systems for Her-2/neu targeted therapies.

Amplification of immune reactivity to Her-2 by depleting regulatory T cells (Tregs)

The weak response of Her-2 transgenic mice to DNA vaccine may result from a reduced T cell receptor repertoire via negative selection in the thymus or suppression of immune reactivity by peripheral regulatory cells. With reports documenting suppressive activity of $CD4^+CD25^+$ Tregs in autoimmune diseases [22], we tested the effect of Treg depletion on tumor immunity. In the initial experiment, BALB/c mice were injected i.p. with CD25 mAb, PC61, either 5 and

6 days before or 1 and 3 days after s.c. inoculation with TUBO or D2F2/E2 cells. D2F2/E2 cells were hormone-induced BALB/c mammary tumor cells stably transfected with human Her-2. Tumors developed in all mice, but >90% of the tumors in Treg depleted mice regressed completely, even after they reached the size of 180 mm^3 [13, 15]. This dramatic tumor regression was induced simply by depleting Tregs, demonstrating in situ priming by a growing tumor. This finding challenges the long-standing paradigm that solid tumors are poor at priming the immune system, thus cannot be rejected effectively by immune cells. Rather, the immunogenicity of a tumor can be functional and powerful when the mice are free of negative regulation.

To test if anti-Her-2 immunity in tolerant mice was also under the control of Tregs [13–15], Her-2 transgenic mice were treated with anti-CD25 mAb 1 week before DNA electro-vaccination, then boosted three times every 2 weeks with the same DNA. Reduction in $CD4^+CD25hi$ cells in the peripheral blood was verified by flow cytometry. The levels of Her-2 Abs were enhanced from undetectable in mice with intact Tregs to 28 ± 27 μg/ml in Treg depleted mice. Therefore, Tregs suppressed Her-2/neu reactivity whether Her-2 was a foreign or self-antigen.

Antibodies (Abs) induced by Her-2 or neu DNA recognized only cognate antigen

After Her-2 and neu transgenic mice were both established, the activity of autologous versus heterologous Her-2/neu vaccines were tested. Wild type, Her-2 or neu transgenic mice were immunized with either human Her-2 or rat neu DNA vaccines. In normal BALB/c and C57BL/6 mice, immunization with Her-2 or neu DNA induced antigen specific IgG which did not cross-react with non-cognate Her-2 or neu [16]. This non-cross-reactive humoral response held true in Her-2 and neu transgenic mice [16]. Vaccination of Her-2 transgenic mice with self Her-2, but not neu induced anti-Her-2 Abs. Similarly, vaccination of NeuT transgenic mice with self neu but not heterologous Her-2 DNA induced anti-neu Abs.

T cells activated by Her-2 or neu DNA exhibited limited cross-reactivity

Compared to humoral response, T cell response induced by heterologous Her-2/neu was more promiscuous. After neu DNA vaccination, BALB/c mice generated T cells that recognized both Her-2 and neu and they rejected D2F2 tumors expressing either Her-2 or neu, showing T cell cross-reactivity when Her-2 and neu were foreign antigens. When NeuT mice were immunized with heterologous pE2TM, splenocytes reacted strongly to human Her-2 and moderately to self rat neu, with anti-neu reactivity at comparable or lower level than that induced by autologous neu DNA vaccine. Conversely, when Her-2 transgenic mice were immunized with heterologous pneuTM, neu-reactive T cells were generated, but they did not recognize autologous Her-2. Therefore, heterologous Her-2/neu vaccines induced Ab to cognate antigen only and induced T cells with varying levels of cross-reactivity to autologous antigens.

In NeuT mice, autologous pneuTM immunization induced T cells that reacted with neu expressing cells, but not the dominant neu epitope, N63, indicating a reduced or altered T cell repertoire in the tolerant host. This finding is consistent with the reported recognition of sub-dominant epitopes [23] or the presence of low avidity CD8 T cells [24] in the immunized FVBneuN transgenic mice. Similarly, CEA transgenic mice also recognized sub-dominant CEA epitopes and this was associated with the expression of CEA in thymic epithelial cells [25].

There have been a number of unidirectional studies on human Her-2 vaccines in rat neu transgenic mice. Immunization of NeuT mice with an adenoviral vector encoding Her-2 induced low-level cross-reactive Abs to neu and delayed tumor formation [26]. The adenoviral vector may induce a broader spectrum of Abs than naked DNA, thus the modest cross-reactivity with neu. In FVBneuN mice, spontaneous tumorigenesis was reduced by immunization with Her-2 DNA every month for 10 months [27]. It is possible that 10 monthly vaccinations with heterologous Her-2 induced enough cross-reactive T cells to autologous neu to exert anti-tumor effect. Tegerstedt et al. [28] reported delay of spontaneous tumorigenesis in BALB NeuT mice by immunization with a single dose of virus-like particle (VLP) displaying heterologous Her-2. It was unclear if cross-reactive Ab or T cells mediated the activity. In most reported vaccine studies including our own, spontaneous tumorigenesis in NeuT mice was controlled primarily by Ab, because NeuT tumors were highly sensitive to Ab even when T cell activity was too low to make an impact [16]. The sensitivity of NeuT tumors to anti-neu antibodies may reflect their exquisite dependence on neu-mediated signals for survival and proliferation.

Her-2/neu model systems recommended for vaccine studies

The limited and variable cross-reactivity between Her-2 and neu responses highlights the importance of using human Her-2 transgenic mice to evaluate human Her-2 vaccines in pre-clinical studies. Rat neu transgenic mice are valuable for establishing the principles of Her-2/neu targeted therapy, but should be used with caution in human vaccine development. Furthermore, all Her-2/neu positive tumor cells are not equally sensitive to Ab treatment. With the increasing use of Her-2 targeted drugs such as Herceptin or tyrosine kinase inhibitors, the surviving tumor cells

may become more resilient, requiring stronger immune modulation to exert anti-tumor immunity.

Phase B: "balance" tumor immunity with autoimmunity

Autoimmune thyroiditis

To address autoimmune side-effects during cancer immunotherapy, the induction of experimental autoimmune thyroiditis (EAT), a model of Hashimoto's thyroiditis, was chosen as the indicator of inadvertent autoimmunity. Hashimoto's thyroiditis, a hypothyroid syndrome, is one of the most prevalent autoimmune manifestations in humans, with 45% of women and 20% of men in USA showing focal thyroiditis at routine autopsy, although only 1% of women and 0.5% of men exhibit clinical symptoms. It is characterized by mononuclear cell infiltration and destruction of the thyroid, elevation of thyroid-stimulating hormone and decrease of thyroid hormones (T3 and T4). The production of autoantibodies to thyroglobulin and thyroid peroxidase [29], and T cell activation by thyroid antigens [30] are indicators of autoreactivity. Susceptibility to thyroiditis is strongly influenced by the haplotype of class II MHC [31]. For example, human HLA-DRB1*0301 (DR3) or murine H2k confers susceptibility to autoimmune thyroiditis [32, 33], whereas C57BL/6 H2b and BALB/c H2d are associated with resistance [34]. DR3, expressed in 24% of normal individuals, is also associated with type 1 diabetes [35] with approximately 20% of type 1 diabetic patients also developing autoimmune thyroid disease [36]. In susceptible individuals, clinical symptoms of thyroiditis are precipitated by endogenous or environmental factors, such as hormone stimulation, dietary iodide level, infection, irradiation, immunostimulatory cytokines, etc., some of these factors are consequences of cancer immunotherapy.

EAT is a well-studied mouse model and is inducible by immunization with mouse thyroglobulin (mTg), the 660 kD storage protein for iodinated thyroid hormone. To simulate humans with circulating thyroid antigens and experiencing various triggering factors, mouse EAT is induced by injecting mTg with an adjuvant such as LPS, or by repeated injections of soluble mTg [15, 37, 38]. Although thyroiditis patients can be treated effectively by hormone replacement therapy, EAT serves as a well-defined experimental system for mechanistic analysis and is a sensitive read-out of autoimmune complications in cancer therapy [39–41].

Tregs in EAT

Removal of suppressor T cells by early thymectomy and/or irradiation leading to low incidence of autoimmune diseases, including thyroiditis and gastritis, was shown in the mid-70s and $CD4^+$ T cells were shown to mediate tolerance in EAT [42]. The CD25 marker enables the separation of Tregs [43, 44]. Removal of $CD25^+$ Tregs enabled EAT resistant mice to develop thyroiditis upon stimulation and abrogated induced tolerance to EAT [15, 44, 45]. Other immune modulating agents, such as antagonist anti-CTLA-4 (Kong, unpublished), agonist anti-CD137 [44] or anti-GITR mAb [46] interfered with the establishment of EAT tolerance. During long-term cancer immunotherapy with repeated blockade of Tregs and/or co-stimulation of T effctors, different autoimmune diseases could manifest themselves [47–52].

Concurrent induction of tumor immunity and autoimmunity by Treg depletion

In BALB/c mice inoculated with TUBO tumor, treatment with anti-CD25 mAb induced tumor regression and neu specific T cells [15]. When the same mice received mTg by chronic i.v. injections, they produced mTg specific Ab and T cells with inflammatory infiltration in the thyroids [15]. The immune responses to neu or mTg were greater in mice undergoing TUBO tumor rejection and mTg injection than in those experiencing either alone. This was the first experimental study to assess amplification of tumor immunity and possible exacerbation of auto-reactivity. To further analyze the concurrent immune responses to Her-2/neu and mTg, we examined genetic regulation of the response in Her-2 tolerant mice.

Genetic regulation of tumor immunity and autoimmunity

A mouse model that expressed both mTg and human Her-2 as self-antigens and expressed HLA-DR3 was established to query the development of EAT during Her-2 vaccination. Heterozygous C57BL/6 Her-2 transgenic mice were mated with homozygous $DR3^+$Abo mice (provided by Dr Chella David, Mayo Clinic, Rochester, MN), which expressed the HLA-DR3 transgene in the absence of endogenous mouse MHC II. The pups were either Her-2^+DR3^+ or Her-2^-DR3^+. The parental B6 Her-2 transgenic mice and the Her-2^+DR3^+ F1 mice both expressed Her-2 as a self-antigen, but with or without DR3, which dictated susceptibility to EAT. IAb of C57BL/6 mice which lack IE, mediated a resistant phenotype to EAT [32]. It has been shown in F1 mice that the parental susceptibility allele was dominant over parental resistant allele [53]. DR3 was expressed in ~20% of DR3Abo PBL and in 10–15% of F1 PBL.

Mice	C57BL/6 Her-2	X	DR3Ab^o	=	Her-2^+xDR3	+	Her-2^-xDR3
Her-2 Ag	self		---		self		---

To measure immune reactivity to Her-2 DNA vaccine, mice were electro-vaccinated with pE2TM and pGM-CSF encoding the extracellular and transmembrane domains of

Her-2 and the murine GM-CSF, respectively. To induce EAT, mice received mTg i.v. with or without LPS. Depletion of Tregs enhanced immune reactivity to Her-2 as well as mTg, verifying control of both Her-2 and mTg responses by Tregs [18]. Her-2^+ × DR3 and Her-2^- × DR3 mice expressing H2b × DR3 haplotype developed more profound mTg response and thyroid pathology than Her-2 or C57BL/6 mice which expressed the EAT resistant H2b haplotype. On the contrary, Her-2 reactivity was comparable whether mice expressed HLA-DR3 or not. Therefore, induction of Her-2 immunity was independent of HLA-DR3 but development of EAT was dictated by this allele, whereas Tregs control the responses to both self-antigens.

These results, however, cannot be taken to indicate MHC independence in Her-2 reactivity. Our preliminary data with Her-2 transgenic mice in C57BL/6 versus BALB/c background suggested a significant difference in their reactivity to Her-2 DNA vaccination (not shown). Although the susceptibility to autoimmunity can be predicted, in part, by class II MHC haplotype, genetic regulation of reactivity to cancer vaccine remains to be determined.

Phase C: "clinical trials"

Her-2 DNA vaccine trials

Several Her-2 DNA vaccine trials have been initiated. A pilot clinical trial testing pVAX-E2A was conducted at the Karolinska Institute, Stockholm, Sweden, in stage IV breast cancer patients. The trial entitled "Vaccine immunization with nucleic acid coding for the gene Her-2/neu together with low doses GM-CSF (Leucomax*) and IL-2 (Proleukin*) as adjuvant in patients with metastatic breast carcinoma" showed no adverse effect. In industry sponsored trials, V930 encoding Her-2 and CEA, generated by Merck, is tested in a phase I trial "V930 First in Man (FIM) Study". The patients in this study included Stages II, III, IV breast, colon, ovarian or non-small cell lung cancer patients with detectable Her-2 and/or CEA expression (http://clinicaltrials.gov/ct/show/NCT00250419?order = 1). Bavarian Nordic's subsidiary BN ImmunoTherapeutics (BNIT) started a "Phase I trial of a fixed dose of MVA-BN-HER2 following first- or second-line chemotherapy for HER-2-positive metastatic breast cancer" with MVA-BN-HER-2, a highly attenuated non-replicating vaccinia virus, MVA-BN®, engineered to encode a modified form of the Her-2 protein. http://clinicaltrials.gov/ct/show/NCT00485277?order = 1.

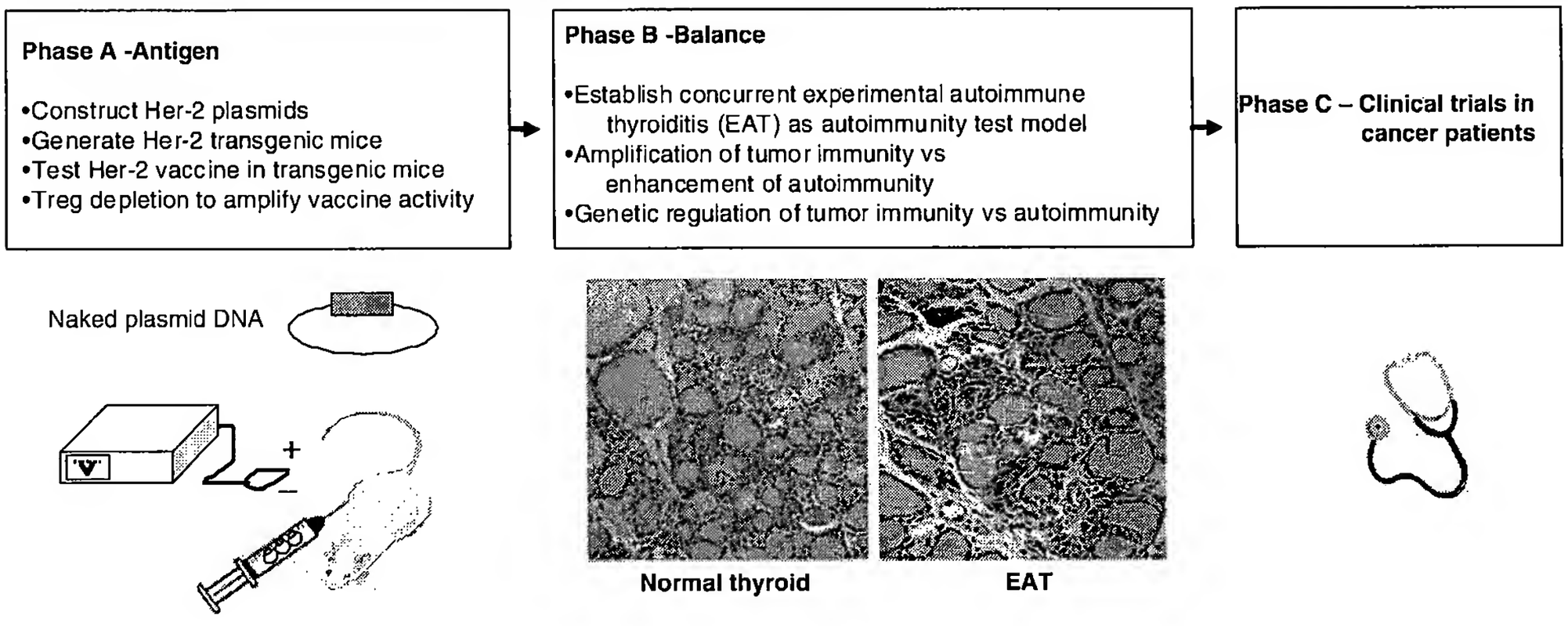
Autoimmune side effects in cancer immunotherapy trials

In cancer patients more strenuous effort than one-time Treg depletion may be required to overcome immune regulatory mechanisms before adequate vaccination efficacy can be induced, with increasing risk of autoimmunity from each new component. Our results showed that even genetically resistant BALB/c mice became susceptible to EAT after Tregs depletion, particularly during tumor regression. Patients expressing high risk HLA haplotypes will require close monitoring of their autoreactivity when their immune regulatory mechanisms are dampened. Severe autoimmune symptoms have already surfaced in cancer immunotherapy trials. In a reported clinical trial testing Her-2 peptide vaccine combined with systemic Flt-3 ligand, 2 of 15 subjects developed elevated thyroid stimulating hormone with symptoms of grade 2 hypothyroidism [39]. In another reported trial, patients with metastatic melanoma received gp100 peptide vaccines along with antagonistic mAb to CTLA-4 [48, 51, 52]. Patients with grade III/IV autoimmune manifestations were observed, including dermatitis, enterocolitis, hepatitis, hypophysitis, hypothyroidism and uveitis. The patients with objective cancer regression all developed severe autoimmune symptoms requiring intervention.

Based on the clinical experience and our findings in EAT model, patients receiving immunomodulating agents, particularly those with susceptible HLA haplotypes, should be closely monitored for autoimmune pathology. Once the importance of balancing tumor immunity with the risk of autoimmunity is recognized, novel strategies can be designed to tilt the balance toward tumor immunity. As an example, direct manipulation of the tumor microenvironment may favor immune priming in situ, thus converting the tumor into a vaccine reservoir without perturbing immune reactivity to most organ-associated self-antigens. This type of strategies to focus immunity to TAA should lead to a safer and more effective vaccine regimen.

Concluding remarks

Her-2 DNA vaccine progressed from a lab innovation to a test agent in clinical trials within a decade. With the available immune modulating tools to complement vaccination, there is optimism that Her-2 vaccine will become a reality. It will be prudent to consider potential side effect at this time, with autoimmunity as the primary concern, and to design counter measures. Although both tumor immunity and autoimmunity are immune responses to self-antigens, the pathology of autoimmunity may not equal the mechanisms of tumor rejection, thus the opportunity to dampen one without compromising the other. It may be further advantageous to deliver immune modulating agents directly into the tumor to exploit the reservoir of tumor antigens in situ, without confronting the entire immune system. Finally, the experience and insight from developing Her-2



vaccine should guide the design of the next vaccine. Surface molecules expressed in abundance and with critical functions, such as Her-2, are desirable targets, whether the molecules are expressed by tumor cells or stromal cells. With the available knowledge and technology, much will be revealed in the next decade and decisive answers to cancer vaccines can be expected.

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EXHIBIT

AQ

A Study to Test V930/V932 in Patients With Cancers Expressing Human Epidermal Growth Factor Receptor 2 (HER-2) and/or Carcinoembryonic Antigen (CEA)

This study is ongoing, but not recruiting participants.

Sponsored by:	**Merck**
Information provided by:	Merck
ClinicalTrials.gov Identifier:	NCT00647114

Purpose

Treatment of patients with cancer types known to express the HER-2 and/or CEA tumor antigens.

Condition	Intervention	Phase
Cancer	Biological: V930 Biological: V932	Phase I

Genetics Home Reference related topics: Benign Tumors Cancer

MedlinePlus related topics: Cancer

ChemIDplus related topics: Epidermal Growth Factor

U.S. FDA Resources

Study Type: Interventional
Study Design: Treatment, Non-Randomized, Open Label, Uncontrolled, Single Group Assignment, Safety Study

Official Title: A Phase I Study to Evaluate the Safety/Tolerability and Immunogenicity of V930/V932 in Patients With **Cancer Expressing HER-2 and/or CEA**

Further study details as provided by Merck:

Primary Outcome Measures:

- To determine the safety and tolerability of V930/V932 followed by EP in cancer patients [Time Frame: Week 22]
  [Designated as safety issue: Yes]

Secondary Outcome Measures:

- To determine whether V930/V932 can elicit HER-2 specific and CEA specific immune responses measured using an ELISPOT assay
  [Time Frame: Week 22] [Designated as safety issue: No]

Estimated Enrollment:	41
Study Start Date:	July 2007
Estimated Primary Completion Date:	December 2008 (Final data collection date for primary outcome measure)

Arms	Assigned Interventions
1: Experimental V930	Biological: V930 V930 - Over a 94 week duration, patients will receive a series of 5 injections (2.5 mg/injection), one every other week. Within 2 minutes of each injection of V930, each patient will be given an EP-IM injection consisting of two 60 msec pulses.
2: Experimental V932	Biological: V932 V932 - Over a 94 week duration, patients will receive a series of 5 injections, 6 patients will initially received intramuscular V932 vaccinations at a low dose (0.5x109 vg/injection),and following a safety assessment, up to an additional 35 patients will be treated with the high dose V932 (0.5x1011 vg/injection).

Eligibility

Ages Eligible for Study:	18 Years and older
Genders Eligible for Study:	Both
Accepts Healthy Volunteers:	No

Criteria

Inclusion Criteria:

- Patients must have completed surgical treatment for his/her primary disease at least 1 month prior to enrollment
- Patient must not be pregnant 3 days prior to enrollment

Exclusion Criteria:

- Patient is currently participating or has participated in a study with an investigational compound or device within 30 days of signing informed consent
- Patient has had their spleen removed or has a history of autoimmune disorders
- Patient is a regular user of any illicit drugs or has used within the past year

of drug or alcohol abuse

- Patient is pregnant or breastfeeding or is expecting to conceive anytime following the study
- Patient is known to be Human Immunodeficiency Virus (HIV)-seropositive
- Patient has a known history of Hepatitis B or C
- Patient has received a vaccine for any disease or condition within one month of enrollment
- Patient has a primary central nervous system tumor.

Contacts and Locations

Please refer to this study by its ClinicalTrials.gov identifier: NCT00647114

Sponsors and Collaborators

Merck

Investigators

Study Director: Medical Monitor Merck

More Information

Responsible Party:	Merck & Co., Inc. (Executive Vice President, Clinical and Quantitative Sciences)
Study ID Numbers:	2007_671, V930-003
First Received:	March 26, 2008
Last Updated:	March 28, 2008
ClinicalTrials.gov Identifier:	NCT00647114
Health Authority:	United States: Food and Drug Administration

Keywords provided by Merck:
Cancers expressing HER-2 and/or CEA

ClinicalTrials.gov processed this record on July 11, 2008

Links to all studies - primarily for crawlers